

# **APPENDIX A**

**(CLEAN VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS)**

**(Serial No.: 09/857,518)**

## TITLE OF THE INVENTION

### FRUIT FLAVOUR RELATED GENES AND USE THEREOF

**[0001]** The invention disclosed herein relates to strawberry (*fragaria ananassa*) and lemon (*citrus limon*) fruit derived genes and enzymes specifically involved in the formation of aliphatic and/or aromatic esters and other aroma and flavour compounds in fruits. More specifically, it relates to a process for improving natural volatile fruit flavours by the control of one or more than one gene implicated in that process.

### BACKGROUND OF THE INVENTION

**[0002]** Like most natural products, the chemical composition of strawberry aroma and flavour is quite complex (Zabetakis and Holden, (1997); J. Sci. Food. Agric. 74: 421-434). Over 300 compounds have been identified which may contribute to flavour/aroma. Qualitatively, the major components of strawberry flavour and aroma may be grouped into several chemical classes including acids, aldehydes, ketones, alcohols, esters, and lactones. Other groups include sulphur compounds, acetals, furans, phenols and even traces of epoxides, and hydrocarbons. Compounds produced by these groups, while often present at low levels, may have a significant impact on the overall flavour of strawberry.

**[0003]** In general, fruit flavour compounds develop during ripening when the metabolism of the fruit changes to catabolism. Esters are proposed to be qualitatively and quantitatively the most important class of volatiles produced and are a key component of strawberry flavour. Seven volatile esters, ethyl hexanoate, methyl hexanoate, ethyl butanoate, methyl butanoate, hexyl acetate, ethyl propionate and 2-hexenyl-acetate were reported to contribute largely to the aroma associated with the fruit. However, more than a 100 types of esters have been reported during the years to be identified in strawberry volatile analysis (Zabetakis and Holden, (1997); J. Sci. Food. Agric. 74: 421-434).

[0004] In lemon, the acetate esters derived from cyclic terpene alcohols are used frequently as flavour, fragrance, scent and aroma components. The lower fatty acid esters of acyclic terpene alcohols (geraniol, linalool, and citronellol) are the acetates (linalyl acetate, geranyl acetate) which are the main flavour components of lemon peel oils. These are used for obtaining citrus notes (Bauer, K. and Garbe, D., (1985). Common fragrance and flavour materials. Preparation, properties and uses. VCH Publishers, Florida, USA). Formates, propionates, and butyrates occur less frequently. In addition to the cyclic and acyclic terpene esters, lemon is a rich source for the ester methyl anthranilate, which is a methyl ester of o-aminobenzoic acid. This compound is known to be a major constituent of *citrus limon* fruit and flowers and can also be found in the leaves.

[0005] Primary metabolites which include sugars, fatty acids, nucleotides and amino acids have been proposed to serve as precursors for the production of secondary metabolites such as volatile compounds. Sugars in the fruit, in addition to acting as a carbon source, act as precursors for the flavour compound furanones (Zabetakis and Holden, (1996); Plant Cell Tissue and Organ Culture 45:25-29). Amino acid metabolism leads to the formation of various aldehydes, acids, carbonyls, and aliphatic and branched-chain alcohols, some of which serve as aroma and flavour components. The changes in amino acid content during maturation of fruits in relation to fruit flavour formation have been studied in banana, tomato and strawberry. The amino acids valine, leucine and isoleucine have been proposed to serve as flavour precursors in banana fruit, and are metabolized to branched chain alcohols, 2-propanol, isoamyl alcohol, and 2-methylbutanol, respectively (Drawert and Berger, (1981); Bioflavour (1981); Schreier, P., Ed.; de Gruyter: Berlin). The conversion of alanine, leucine and valine to flavour compounds has also been demonstrated in tomato (Yu *et al.*, (1968); Phytochemistry 6: 1457-1465). In strawberry, alanine was proposed to be the main free amino acid metabolized to flavour compounds. This was based mainly on the dramatic decrease in its content just before the formation of volatile aroma and flavour compounds commenced in the fruit (Perez *et al.*, (1992); J.Agric. Food Chem. 40: 2232-2235). In addition, feeding alanine to strawberry cultures resulted in the formation of several esters such as methyl and ethyl hexanoate which are important constituents of strawberry volatiles (Drawert and Berger, (1981); Bioflavour (1981); Schreier, P., Ed.; de Gruyter: Berlin). Like sugars and amino acids, the fatty acids in strawberry play an important role in ester formation. In mango fruit there have been reports that fatty acids are

actively metabolized during ripening. Changes in fatty acids and triglycerides have been associated with changes in aroma and flavour during mango ripening (Gholop A.S. and Bandyopadhyay C., J. Am. Oil Chem. Soc., 52: 514-516, 1975). Therefore, fatty acid-related enzymes may have a role in the production of flavour volatiles. Thiolase is the last enzyme in the  $\beta$ -oxidation of fatty acids. It catalyzes the thiolytic cleavage involving a molecule of CoA. The products of this reaction are acetyl-CoA and acyl-CoA derivatives containing two carbon atoms less than the original acyl-CoA molecule that underwent oxidation. The acyl-CoA formed in the cleavage reaction may be utilized at the final stage of the biosynthetic pathway for ester formation in fruit. Therefore, the profile of fatty acid and amino acid precursors found in each fruit, along with the specificity of the enzymes in the biosynthetic pathway/process leading to ester formation, could have a key role in determining the types of esters formed.

**[0006]** A proposed biosynthetic pathway for volatile ester formation in fruits is illustrated in Figure 1. In fruits, the transamination and oxidative decarboxylation of amino acids provides the precursors for volatile aroma compounds such as aldehydes, acids, alcohols, esters and thiols. The transamination reaction is catalyzed by aminotransferases. Aminotransferases or transaminases are enzymes promoting the first step in the catabolism of L-amino acids by removing the  $\alpha$ -amino groups. As a result of this transamination reaction, the  $\alpha$ -amino group is transferred to the  $\alpha$ -carbon atom of  $\alpha$ -ketoglutarate, leaving behind the  $\alpha$ -keto acid analogue of the amino acid. Aminotransferases have been studied in various microorganisms and some of them have been purified (Gelfand *et al.*, (1997); J. Bacteriol. 130:429-440, Lee *et al.*, (1985); J. Gen. Microbiol. 131: 459-467). No strawberry fruit specific aminotransferase to date has been cloned or characterized.

**[0007]** Ketoacids produced by transamination can be enzymatically degraded to the corresponding aldehydes or carboxylic acids. The enzyme which can catalyze this reaction is pyruvate decarboxylase. An EST encoding part of pyruvate decarboxylase, the first to be characterized from a strawberry fruit, has been mentioned in patent application WO 97/27295. The pyruvate decarboxylase enzyme provides precursors for the biosynthesis of volatile flavour compounds. Alcohol dehydrogenase enzymes have been implicated in the interconversion of the aldehyde and alcohol forms of flavour volatiles. The mechanism of ester formation has been shown to be a coenzyme A dependent reaction. Esterification is a result of transacylation from acyl-coen-

zyme A to an alcohol. The enzyme is, therefore, termed as alcohol acyl transferase (AAT) and plays a major role in the biosynthesis of volatile esters.

**[0008]** It is still unclear whether esterase enzymes (Fig. 1) are involved in the hydrolysis of esters or in their synthesis (Mauricio *et al.*, (1993) J. Agric. Food Chem. 41, 2086-2091).

**[0009]** Ester formation by microorganisms has been studied in most detail and has been cited in a number of publications (Harada *et al.*, (1985); Plant Cell Physiol. 26(6): 1067-1074), Fujii *et al.*, (1996); Yeast 12:593-598, Fujii *et al.*, (1994); Applied and Enviro. Microbiology 8:2786-2792 ). Alcohol acyl transferases (AAT's) have been identified from both fungi (Yoshihide *et al.*, (1978); Agric. Biol. Chem. 42(2):269-274) and yeast (Fujii *et al.*, (1994); Applied and Enviro. Microbiology 8:2786-2792) and have been the subject of patent applications (EP 0 574 941 A2). The AAT from the fungi *Neurospora* sp. which was purified to homogeneity acts on various acyl coenzyme A containing more than a four carbon linear chain but not on acetyl coenzyme A as described for the yeast enzyme (Yamauchi *et al.*, (1989); Agric. Biol. Chem. 53(6) 1551-1556). In *Cladosporium cladosporioides* No. 9, AAT was partially purified and was described to form acetate esters like the yeast enzyme (Yamakawa *et al.*, (1978); Agric. Biol. Chem. 42(2):269-274). So it is clear that the type of esters formed is dependent on the substrate specificity of the ester-forming enzyme to alcohols and the availability of acyl-CoAs. For example, the yeast *Saccharomyces cerevisiae* (sake yeast) and brewer's yeast (bottom fermenting yeast) AAT's show high affinity to acetyl-CoA and the alcohols ethanol and isoamyl alcohol, giving rise to the formation of acetate esters. These esters play an important role in determining flavour characteristics of beer.

**[0010]** In plants, ester formation has been studied both in fruit and flowers. Melon, banana and strawberry AAT's have been investigated using crude fruit extracts. The formation of esters from aldehydes that were incubated with whole strawberry fruit has been reported (Yamashita, *et al.*, (1989); Agr. Biol. Chem. 39(12) 2303-2307). Analysis of the substrate specificity of the enzymes from the various sources, revealed differences in their affinity to acyl-CoAs and alcohols (Ueda *et al.*, (1992); Nippon Shokukin Kogyo Gakkaishi 39(2): 183-187, Perez *et al.*, (1996); J. Agric. Food Chem. 44: 3286-3290, Harada *et al.*, (1985); Plant Cell Physiol. 26(6): 1067-1074). Maximum activity for the strawberry (AAT) was obtained using acetyl CoA and hexyl alcohol as substrates, and acetyl-CoA and butyl alcohol is the preferred substrate for the banana enzyme. A

clear correlation could be observed between substrate preference and volatile esters present in both fruits. The purification method and some properties of the strawberry AATase enzyme have been reported. However, to date, no sequence of the peptide or of the gene has been disclosed. In *Clarkia breweri*, the ester benzylacetate is an important constituent of flower scent. The purification of the protein and the gene AAT encoding it has been reported. The flower AAT enzyme was not reported to have affinity to aliphatic alcohols. The enzyme has high affinity to the aromatic alcohols such as benzyl alcohol and cinnamyl alcohol (Dudareva *et al.*, (1998); *The Plant Journal* 14(3) 297-304). Genes coding for flower aromatic acyl transferases that acylate plant pigments, causing changes in color tone, have been the subject of a recent patent application (EP 0810287). Recently, an aromatic amino transferase enzyme has been purified from lactic acid bacteria and was shown to initiate the conversion of amino acids to cheese flavour compounds (Yvon *et al.*, (1997); *Applied and Environmental Microbiology* 414-419).

**[0011]** Although volatile esters are qualitatively and quantitatively one of the most important classes of volatile compounds in fruit flavour and aroma, there are very few reports concerning the biochemical aspects of ester formation in fruits. An understanding of the precursors and characterization of enzymes involved in the pathways leading to the formation of flavours in fruit is essential for the production of natural flavours. Plant-derived flavour components alone represent a world-wide market of 1.5 billion dollars. Presently, the main way to produce plant flavour compounds is by the synthetic route. Synthetic organic chemicals constitute more than 80-90% (by weight and value) of the raw materials used in flavour and fragrance formulations. Problems often exist concerning production. Extraction from intact plants and conventional fermentation are currently providing alternative routes for the commercial production of flavour and aroma chemicals. However, the demand for natural flavours by the consumer has been steadily increasing, and often demand outstrips supply. In many cases, sought after flavour compounds preclude isolation. Unlike other fruit flavours, no single chemical or class of chemicals in particular is associated with strawberry flavour. To date, no strawberry fruit- specific aminotransferase, pyruvate decarboxylase, thiolase, alcohol dehydrogenase, or acyl-transferase enzymes at the route of this invention, involved directly or indirectly in providing precursors for ester formation, hence flavour compounds, has been isolated and characterized. The genes and their peptides at the route of this invention are involved in the

biosynthetic pathway for aliphatic and/or aromatic ester production in fruit and can, therefore, provide a novel method for the *in-vivo* and *in-vitro* biotechnology production of bioflavours, natural flavour chemicals by recombinant means.

## SUMMARY OF INVENTION

**[0012]** The object of the present invention is to disclose the genes/peptides involved in the process of volatile ester production, particularly aliphatic esters although not exclusively so, hence fruit flavour and aroma. This is the first time to our knowledge that the gene/peptide sequences from an entire pathway to the production of volatile esters in fruit, in particularly strawberry, have been disclosed. The nucleotide and polypeptide sequences described in this application can serve as a tool for a vast number of applications related to ester formation and the biotechnological engineering of natural and artificial fruit flavours for the food industry. The invention is based on the identification of genes which encode proteins central to the pathway leading to volatile ester formation in fruit. DNA sequences which encode these proteins have been cloned and characterized. The nucleic acid/peptide sequences may be used in expression systems, for industrial application and/or to modify plants with the goal to produce natural and/or synthetic flavours.

## DEFINITIONS OF TERMS

**[0013]** “Nucleic acid” sequence as used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represents the sense or antisense strand. Similarly, “amino acid sequence” as used herein refers to a peptide or protein sequence.

**[0014]** The term “agonist”, as used herein, refers to a molecule which, when bound to a polypeptide, causes a change in the polypeptide which modifies the activity of the polypeptide. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecule which binds to the polypeptide.

**[0015]** The term “antagonist” or “inhibitor” as used herein refers to a molecule which, when bound to a polypeptide, blocks or modulates the biological or immunological activity of the

polypeptide. Antagonists or inhibitors may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to the polypeptide.

**[0016]** “Stringency” typically occurs in the range from about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe) to about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those skilled in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences.

**[0017]** The term “hybridization” as used herein shall include “any process by which a strand of nucleic acid joins with a complementary strand through base pairing” (Coombs J., (1994) Directory of Biotechnology, Stockton Press, New York NY).

**[0018]** A “deletion” is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

**[0019]** An “insertion” or “addition” is that change in nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring polypeptide(s).

**[0020]** A “substitution” results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

**[0021]** As used herein, the term “substantially purified” refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and that are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated. The definition “substantially homologous” means that a particular subject sequence, for example, a mutant sequence, varies from the reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and the subject sequence.

**[0022]** A “variant” of a polypeptide as outlined in the first and second aspects of this invention is defined as an amino acid sequence that is different by one or more amino acid “substitutions”. A variant may have “conservative” changes, wherein a substituted amino acid has similar structural or chemical properties e.g., replacement of leucine with isoleucine. More rarely, a variant may have “nonconservative” changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in

determining which and how many amino acid residues may be substituted, inserted or deleted, without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTar software.

**[0023]** The term “biologically active” refers to a polypeptide as outlined in the first and second aspects of the invention, having structural, regulatory, or biochemical function of their naturally occurring counterparts. Likewise, “immunologically active” defines the capability of the natural, recombinant or synthetic polypeptide, as outlined in the first and second aspects of the invention, or any oligopeptide thereof, to induce a specific immune response in an appropriate animal or cell and to bind with specific antibodies.

**[0024]** The term “natural products” refers to those products that are obtained directly from plants and sometimes from animal sources by physical procedures. “Nature identical compounds” are produced synthetically but are chemically identical to their “natural counterparts”. Artificial flavour substances are compounds that have not been identified in plant or animal products for human consumption. “Nature-identical” aroma substances are, with few exceptions, the only synthetic compounds used in flavours in addition to “natural products”.

**[0025]** The definition of “host cell” refers to a cell in which an alien process is executed by bio-interaction, irrespective of the cell belonging to a unicellular, multicellular, a differentiated organism or to an artificial cell, cell culture or protoplast. The definition of “host cell” in the context of this invention is to encompass the definition “plant cell”.

**[0026]** “Plant cell” by definition refers to any self-propagating cell bounded by a semipermeable membrane and containing one or more plastids. Such a cell requires a cell wall if further propagation is required. “Plant cell”, as used herein, includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callous tissues, protoplasts, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores.

**[0027]** With the definition “transformed cell” or “transgenic cell” is meant a cell (or ancestor of said cell) into which by means of recombinant DNA techniques, DNA encoding the target polypeptide can be introduced.

**[0028]** The definition of “micro-organisms” refers to microscopic organisms, such as Archaea, Bacteria, Cyanobacteria, Microalgae, Fungi, Yeast, Viruses, Protozoa, Rotifers, Nematodes, Micro-Crustaceans, Micro-Molluscs, Micro-Shellfish, Micro-insects, etc.

**[0029]** The definition of “plant(s)” refers to eukaryotic, autotrophic organisms. They are characterized by direct usage of solar energy for their primary metabolism, their permanent cell wall and, in the case of multicellular individuals, their open unlimited growth. In the case of heterotrophic plants, the organisms are, in an evolutionary context, essentially derived from autotrophic plants in their structure and metabolism.

**[0030]** “Dicotyledons” (and all scientific equivalents referring to the same group of plants) form one of the two divisions of the flowering plants or angiospermae in which the embryo has two or more free or fused cotyledons.

**[0031]** “Monocotyledons” (and all scientific equivalents referring to the same group of plants) form one of the two divisions of the flowering plants or angiospermae in which the embryo has one cotyledon.

**[0032]** “Angiospermae” or flowering plants are seed plants characterized by flowers as specialized organs of plant reproduction and by carpels covering the ovaries. “Gymnospermae” are seed plants characterized by strobilus as specialized organs for plant reproduction and by naked sporophylls bearing the male or female reproductive organs. “Ornamental” plants are plants that are primarily in cultivation for their habitus, special shape (flower, foliage or otherwise), color or other characteristics which contribute to human well-being indoors as cut flowers or potted plants or outdoors in the man-made landscape. “Vegetables” are plants that are purposely bred or selected for human consumption of foliage, tubers, stems, fruits, flowers or parts of them and that need an intensive cultivation regime. “Arable crops” are purposely bred or selected for human objectivities (ranging from direct or indirect consumption, feed or industrial applications such as fibers).

**[0033]** The definition of “process” is the development of a method(ology) concerning the progress of a series of activities in a certain context. “Biological” process is a process based on metabolic activity in organisms, or essentially derived from that by biochemical, biophysical, physiological, ecological or genetic means. “Industrial” process is an economically feasible process with high added value, aiming at bulk production.

**[0034]** The definition of “homology” refers to the basic similarity of a particular structure in different organisms, usually resulting from the descent from a common ancestor.

**[0035]** The definition of “promoter” is intended as a nucleotide sequence sufficient to direct transcription. Also included are those promoter elements which are sufficient to render tissue-specific gene expression; such elements may be located in the 5’ or 3’ regions of the native gene.

**[0036]** The definition of “operably linked” is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

**[0037]** The definition of “fruit” (botanically) is the ripened ovary of a plant and its contents. The definition of “fruit” (agronomically, linguistically) is the ripened ovary and its contents together with any structure with which they are combined, as in case of strawberry or apple, the receptacle.

**[0038]** The definition of “climacteric” is pointing at the phase of increased respiration found at fruit ripening and at senescence. The definition “nonclimacteric” is pointing at no such phase being present.

**[0039]** The definition of “antisense” RNA is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or majority of bases) in the antisense strand (read in the 5’ to 3’ sense) is capable of pairing with the corresponding base (G with C, A with U), in the mRNA sequence read in the 5’ to 3’ sense.

**[0040]** The definition of “sense” RNA is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence.

## BRIEF DESCRIPTION OF DRAWINGS

**[0041]** The present invention may be more fully understood by reference to the following description, when read together with the accompanying drawings:

**[0042]** Figure 1. The proposed biosynthetic pathway for the production of esters in fruits.

**[0043]** Figure 2. Volatile ester emission during strawberry fruit development. GC-MS chromatograms (detector response: 100% =  $2 \times 10^6$  total ion counts) of volatiles *in-vivo* released by strawberry fruits (cv. Elsanta) at different stages of development. Developmental stages: G-green;

W-white; T-turning; P- pink; R-red; DR-dark red. The five main volatile esters detected are marked with numbers: 1-methyl hexanoate; 2-hexyl acetate; 3-hexyl butanoate; 4-octyl acetate; 5-octyl butyrate. A GCMS chromatogram of a mature strawberry fruit of the cultivar *Elsanta*. The analysis was done using the headspace method. Esters are marked with dots.

**[0044]** Figure 3. The expression pattern of strawberry alcohol acyl transferase cDNA (SLE27, SEQ ID NO: 1) in various tissues of the cultivar *Elsanta* by Northern blot analysis. The Northern blot was hybridized with a full-length cDNA fragment of SEQ ID NO: 1 (SLE27).

**[0045]** Figure 4. The expression pattern of a lemon alcohol acyl transferase (CLF26, SEQ ID NO: 2) in various tissues of the lemon cultivar *Mayer* by Northern blot analysis. The Northern blot was hybridized with a full-length cDNA fragment of SEQ ID:2 (CLF26).

**[0046]** Figure 5. Expression analysis in various tissues of the strawberry cultivar *Elsanta* by Northern blot analysis. The Northern blots were hybridized with full-length cDNA fragments corresponding to (a) a thiolase SEQ ID NO: 3 (SLG150) (b) a pyruvate decarboxylase SEQ ID NO: 4 (SLH51) and (c) an alcohol dehydrogenase SEQ ID NO: 5 (SLB39).

**[0047]** Figure 6. Strawberry genomic Southern analysis. 10 lg DNA was digested with restriction enzymes HindIII, EcoRI, XbaI and XhoI, separated on an agarose gel and blotted onto a nylon filter. The DNA was hybridized with a full-length <sup>32</sup>P-labeled strawberry alcohol acyl transferase cDNA (SLE27) probe, corresponding to SEQ ID NO: 1 and autoradiographed.

**[0048]** Figure 7. A scheme of the pRSET B (Invitrogen) vector used for the expression of various alcohol acyl transferases in *E.coli*.

**[0049]** Figure 8. Verification of ester formation by the SLE27 (SAAT), SEQ ID NO: 6 protein using GC-MS. GC-MS chromatograms (detector response: 100% = 2x10<sup>6</sup> total ion counts) of volatiles produced with the incubation conditions as described below. **(A)** Butanol and butyl acetate standards. **(B)** SAAT protein + butanol + acetyl-CoA. **(C)** As in B, protein absent. **(D)** As in B, butanol absent. **(E)** As in B, acetyl-CoA absent. **(F)** GFP protein + butanol + acetyl-CoA. **(G)** Empty pRSET B vector eluate + butanol + acetyl-CoA. Other visible peaks are impurities from the butanol substrate.

**[0050]** Figure 9. Western blot loaded with protein corresponding to four different treatments carried out on the pellet, supernatant and eluate from a Ni-NTA column after expression

of strawberry alcohol acyl transferase (SLE 27, SEQ ID NO: 1) in *E. coli*. As a control for the experiment, the Green Fluorescent Protein (GFP) was partially purified in the same way and the eluate from one treatment was loaded on the gel. The blot was hybridized with a commercial antibody (Clontech) raised against an epitope fused at the N- terminus of the recombinant protein.

**[0051]** Figure 10. Gene expression profiles of strawberry alcohol acyl transferase (SLE 27, SEQ ID NO: 1), alcohol dehydrogenase (SLF138, SEQ ID NO: 7), pyruvate decarboxylase (SLH 51, SEQ ID NO: 4), alcohol dehydrogenase (SLG16, SEQ ID NO: 8) and amino transferase (SLF96, SEQ ID NO: 9). Expression ratios were monitored during fruit development and ripening in the red stage vs. green fruit and red fruit vs. turning fruit.

**[0052]** Figure 11. SLE27 (SAAT), SEQ ID NO: 6 (SAAT)-induced ( $^{14}\text{C}$ )-acetyl-CoA incorporation in esters. **(A)** Flame ionization detector (FID)-signal of unlabeled standards of **1**, hexylacetate; **2**, 1-hexanol; **3**, octylacetate and **4**, 1-octanol. **(B)** and **(C)**: Radio-GC chromatograms of labeled products formed by the SAAT protein from 0.1 mM ( $^{14}\text{C}$ )-acetyl-CoA and alcohols with **(B)** 2 mM 1-octanol or **(C)** 2 mM 1-hexanol.

**[0053]** Figure 12. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with 1-butanol.

**[0054]** Figure 13. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the melon alcohol acyl transferase (MAY 5, SEQ ID NO: 10). **(B)** *E. coli* cell cultures expressing the empty vector cassette. No alcohol supplements.

**[0055]** Figure 14. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the apple alcohol acyl transferase (MAY 3, SEQ ID NO: 11). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with 1-butanol.

**[0056]** Figure 15. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the citrus alcohol acyl transferase (CLF26: SEQ ID NO: 2). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with 1-butanol.

**[0057]** Figure 16. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the banana alcohol acyl transferase (MAY 2, SEQ ID NO: 12). **(B)** *E. coli* cell cultures expressing the empty vector cassette. No alcohol supplements.

**[0058]** Figure 17. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the apple alcohol acyl transferase (MAY 3, SEQ ID NO: 11). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with isoamylalcohol, n-butanol, cis-2-hexen-1-ol and isopropanol.

**[0059]** Figure 18. Verification of ester formation using GC-MS **(A)** *E. coli* cell cultures expressing the strawberry vesca alcohol acyl transferase (SUN1, SEQ ID NO: 13). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with isoamylalcohol, n-butanol, cis-2-hexen-1-ol and isopropanol.

#### DETAILED DESCRIPTION OF THE INVENTION

**[0060]** According to a first aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 6 or SEQ ID NO: 14, or
- (b) a polypeptide having at least 30% homology with the amino acid sequence SEQ ID NO: 6 or at least 40% homology with the amino acid sequence SEQ ID NO: 14, or
- (c) a fragment of polypeptide (a) or (b),

which polypeptide or fragment of polypeptide has alcohol acyl transferase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0061]** Preferably, the DNA sequence encodes a polypeptide having at least 50% homology, more preferably at least 70% homology, with any of the amino acid sequences SEQ ID NO: 6 or SEQ ID NO: 14, or a fragment thereof.

**[0062]** With reference to the nucleic acid sequence, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 1 or SEQ ID NO: 2 which encodes a polypeptide having alcohol acyl transferase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 1 or SEQ ID NO: 2, or
- (c) which has at least 25% homology with any of the sequences of (a) or (b), or

- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a)-(c).

Preferably, the DNA sequence has a nucleic acid sequence

- (e) which has at least 40% homology, more preferably at least 60% homology, with any of the sequences of (a) or (b), or
- (f) which is capable of hybridizing under stringent conditions to said sequence of (c).

**[0063]** According to a second aspect, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 6 or SEQ ID NO: 14, or
- (b) having at least 30% homology with the amino acid sequence SEQ ID NO: 6 or at least 40% homology with the amino acid sequence SEQ ID NO: 14, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has alcohol acyl transferase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0064]** Preferably, the polypeptide has at least 50% homology, more preferably at least 70% homology, with any of the amino acid sequences SEQ ID NO: 6 or SEQ ID NO: 14, or a fragment thereof.

**[0065]** It may be advantageous to produce nucleotide sequences according to the first aspect of the invention as defined above or derivatives thereof possessing a substantially different codon usage. It is known by those skilled in the art that, as a result of degeneracy of the genetic code, a multitude of gene sequences, some bearing minimal homology to the nucleotide sequences of any known and any naturally occurring genes, may be produced. The invention contemplates each and every possible variation of the nucleotide sequences that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring gene sequences, and all such variations are to be considered as being specifically disclosed. In addition, the nucleotide sequences of the invention may be used in molecular biology techniques that have not been developed, providing the new techniques rely on properties of nucleotide sequences that are currently

known, including but not limited to properties such as the triplet genetic code and specific base pair interactions.

**[0066]** Altered nucleic acid sequences of this invention include deletions, insertions, and substitutions of different nucleotides resulting in the polynucleotides that encode the same or are functionally equivalent. Deliberate amino acid substitution may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, and/or the amphipathetic nature of the residues as long as the biological activity of the polypeptide is retained. Included in the scope of the present invention are alleles of the polypeptides according to the second aspect of the invention as defined above. As used herein, an “allele” or “allelic sequence” is an alternative form of the polypeptides described above. Alleles result from a mutation, e.g., a change in the nucleic acid sequence, and generally produce altered mRNA or polypeptides whose structure or function may or may not be altered. Any given polypeptide may have one, or more allelic forms. Common allelic changes that give rise to alleles are generally ascribed to natural deletions, additions or substitutions of amino acids. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

**[0067]** It is envisaged that the polynucleotide sequences of the present invention can be used as probes for the isolation of similar sequences from the strawberry genome and lemon genome. By using a cDNA of the gene sequences of the invention as a probe, it would be possible by those skilled in the art to obtain comparable gene sequences from various tissues, particularly fruit tissues, from climacteric and nonclimacteric plants. One aspect of the invention is to provide for hybridization or PCR probes which are capable of detecting polynucleotide sequences, including genomic sequence(s), encoding the polypeptides of the invention, or closely related molecules. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g. in the 3' region, and the stringency of the hybridization or amplification (maximal, high, intermediate, low) will determine whether the probe identifies only naturally occurring sequence(s) encoding the polypeptide, allele's or related sequences.

**[0068]** Additional alcohol acyl transferase sequences from melon, strawberry, vesca, banana, apple, mango and lemon were obtained using primers. These additional sequences are covered by the first aspect of invention. The nucleic acid sequences are shown in SEQ ID NOS:10,

11, 12, 13, 15, 16, 17, and 18. The amino acid sequences are shown in SEQ ID NOS:19 to 26. Apart from the applications as disclosed herein, these sequences can be used in combination for the synthesis of a broader range of esters via recombinant means or via transgenic approaches and/or for the synthesis of new flavours.

**[0069]** Probes may also be used for the detection of related sequences and preferably contain at least 50% of any of the nucleotides from any one of the gene-encoding sequences according to the present invention. For gene sequences of the present invention, the hybridization probes may be derived from the nucleotide sequence, or from genomic sequences including promoter, enhancer element and introns. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , or enzymatic labels such as alkaline phosphatase coupled to the probe *via* avidin/biotin coupling systems, and the like.

**[0070]** Other means for producing specific hybridization probes for encoding gene sequences of the invention include the cloning of nucleic acid sequences into suitable vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes *in-vitro* by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriately radioactively labeled nucleotides.

**[0071]** According to a third aspect, the invention provides a recombinant expression vector comprising a coding sequence which is operably linked to a promoter sequence capable of directing expression of the coding sequence in a host cell for the vector and a transcription termination sequence, in which the coding sequence is a DNA sequence according to the invention.

**[0072]** In a further embodiment, the invention provides a replicative cloning vector comprising an isolated DNA sequence of the invention and a replicon operative in a host cell for said vector.

**[0073]** Methods which are well known to those skilled in the art can be used to construct expression vectors containing the gene coding sequence of the invention and appropriate transcriptional and translational controls. These methods include *in-vitro* recombinant techniques. Such techniques are described in Sambrook *et al.*, 1989. Molecular cloning a laboratory manual, Cold Spring Harbour Press, Plain View, NY and Ausubel F.M. *et al.*, (1989). Current protocols in molecular biology, John Wiley and Sons, New York, NY.

**[0074]** In order to express a biologically active polypeptide, the nucleotide sequence encoding the polypeptide, or the functional equivalents or fragments thereof is inserted into the appropriate expression vector (i.e., a vector that contains the necessary elements for the transcription or translation of the inserted coding sequence). Specific initiation signals may also be required for efficient translation of the polypeptides of the invention. These signals include the ATG initiation codon and adjacent sequences. In cases where the polypeptides, their initiation codons and upstream sequences are inserted into the appropriate expression vector, no additional translational control systems including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf D. et al. (1994) *Results Prob. Cell Differ.* 20: 125-62; Bittner *et al.* (1987) *Methods in Enzymol.* 153: 516-544).

**[0075]** In addition, a host strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acylation, carboxylation, glycosylation, phosphorylation and lipidation. Post-translation processing which cleaves a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells which have the correct cellular machinery and characteristic mechanisms for such post-translational activities may be chosen to ensure correct modification and processing of the introduced, foreign protein.

**[0076]** The nucleotide sequences of the present invention can be engineered in order to alter the coding sequence for a variety of reasons, including, but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the state of the art, e.g., site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns, to change codon usage, to produce splice variants, etc. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular procaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host (Murray E. *et al.* (1989) *Nuc. Acids*

Res. 17: 477-508). Other reasons for substantially altering the nucleotide sequence(s) of the invention and their derivatives, without altering the encoded amino acid sequences, include the production of RNA transcripts having more desirable properties, such as a greater half life, than transcripts produced from naturally occurring sequences.

[0077] In developing the expression cassette, the various fragments comprising the regulatory regions and open reading frame may be subjected to different processing conditions, such as ligation, restriction enzyme digestion, resection, *in-vitro* mutagenesis, primer repair, use of linkers and adapters and the like. Thus, nucleotide transitions, transversions, insertions, deletions and the like may be performed on the DNA which is employed in the regulatory regions and/or open reading frame. The expression cassette may be wholly or partially derived from natural sources endogenous to the host cell. Furthermore, the various DNA constructs (DNA sequences, vectors, plasmids, expression cassettes) of the invention are isolated and/or purified, or synthesized and thus are not naturally occurring. The invention further contemplates the use of yet undescribed biological and non-biological-based expression systems and novel host systems that can be utilized to contain and express the gene coding sequence(s) of the invention.

[0078] “Antisense” or “partial sense” or other techniques may also be used to reduce the expression of genes in the pathway leading to the production of flavour volatile esters. Full-length “sense” techniques may be designed to increase or reduce the expression of genes in the pathway leading to the production of flavour volatiles. The “antisense” or “partial sense” molecules may be designed to block translation of mRNA by preventing the transcript from binding to the ribosomes. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of gene sequences of the invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the

oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

**[0079]** “Antisense” molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in-vitro* and *in-vivo* transcription of DNA sequences of the invention. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 and SP6. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells or tissues. RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences, at the 5’ and/or 3’ end of the molecule, or the use of phosphorothioate or 2’ O-methyl rather than phosphodiesterase linkages within the backbone of the molecule.

**[0080]** A variety of vector/host expression systems can be utilized to contain and express the gene coding sequences of the invention. These include and are not limited to micro-organisms such as bacteria (e.g., *E. coli*, *B. subtilis*, *Streptomyces*, *Pseudomonads*) transformed with recombinant bacteriophage, plasmid or cosmid DNA expression systems, yeast (e.g., *S. cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris*, *Hansenula polymorpha*, *Schizosacch. Pombe*, *Yarrowia*) transformed with yeast expression vectors; filamentous fungi (*Aspergillus nidulans*, *Aspergillus orizae*, *Aspergillus niger*) transformed with filamentous fungi expression vectors, insect cell systems transfected with virus expression vectors (e.g., baculovirus, adenovirus, herpes or vaccinia viruses); plant cell systems transfected with virus expression vectors (e.g., cauliflower mosaic virus, CaMV, tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (e.g. Ti or Pbr322 plasmid); or mammalian cell systems (chinese hamster ovary (CHO), baby hamster kidney (BHK), hybridoma’s, including cell lines of mouse, monkey, human and the like.

**[0081]** In the case of plant expression vectors, the expression of a sequence(s) of the invention may be driven by a number of previously defined promoters, including inducible and developmentally regulated promoters. The invention further contemplates the use of the individual

promoters of the polynucleotide sequence(s) of the present invention for this purpose. In particular, an alcohol acyl transferase promoter(s) pertaining to SEQ ID NO: 1 and SEQ ID NO: 2 or any promoters particularly responsive to ripening events may be used to drive the tissue-specific expression of the target genes. In addition, viral promoters such as the 35S and the 19S promoters of CaMV (Brisson *et al.*, 1984; Nature, 310: 511-514) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu *et al.* (1987); *EMBO J* 6:307-311). Promoters or enhancers derived from the genomes of plant cells, tissue-specific promoters, i.e., fruit-specific promoters, Fbp7 (Columbo *et al.* 1997; Plant Cell 9; 703-715), 2A11 promoter (Pear *et al.*, 1989, *Plant Molecular Biology*, 13:639-651), small subunit of Rubisco (Corruzzi *et al.*, 1984; *EMBO J* 3:16; Broglie *et al.*, 1984 Science 224:838-843) or timing-specific promoters such as ripening specific promoters (the E8 promoter, Diekman and Fisher, 1988, *EMBO J*, 7:3315-3320) may be used. Suitable terminator sequences include that of the *agrobacterium tumefaciens* nopaline synthase gene (Nos 3' end), the tobacco ribulose biphosphate carboxylase small subunit termination region, and other 3' regions known to those skilled in the art. These constructs can be introduced into plant cells by direct DNA transformation, or pathogen mediated transfection. For reviews of such techniques, see Hobbs S. or Murry L.E., in McGraw Hill Yearbook of Science and Technology (1992), McGraw Hill NY, pp 191-196, or Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, Academic Press, New York, NY, pp421-463, or Fillatti *et al.*, Biotechnology, 5: 726-730. Manipulation of DNA sequences in plant cells may be carried out using the Cre/lox site-specific recombination system as outlined in patent application WO91-09957.

**[0082]** According to a fourth aspect, the invention provides a method for regulating aliphatic and/or aromatic ester formation in fruit, comprising inserting into the genome of a fruit-producing plant one or more copies of one or more DNA sequences of the invention.

**[0083]** The activity of genes according to the present invention involved in the biosynthetic pathway leading to volatile ester formation, hence flavour, may be either increased or reduced depending on the characteristics desired for the modified plant part. The gene sequence may be in the same "sense" or "antisense" orientation as the endogenous target gene. Methods well known to those skilled in the art can be used to construct recombinant vectors which will express "sense" or "antisense" polynucleotides encoding the gene sequences of the present invention. Such

technology is now well known in the art. In the case of the “plant cell” officiating as “host cell”, the target “plant cell” may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. The target plant may be selected from any monocotyledonous or dicotyledonous plant species. Suitable plants include any fruit-bearing plant such as strawberry, citrus (lemon), banana, apple, pear, melon, sweet pepper, peach or mango. Other suitable plant hosts include vegetable, ornamental (to include sunflower) and arable crops (to include soybean, sunflower, corn, peanut, maize, wheat, cotton, safflower, and rapeseed). For any particular plant cell, the gene sequences, cDNA, genomic DNA, or synthetic polynucleotide, used in the transformation vector construct, may be derived from the same plant species, or may be derived from other plant species (as there will be sufficient homology to allow modification of related enzyme gene function). The procedure or method for preparing a transformant can be performed according to the conventional technique used in the fields of molecular biology, biotechnology and genetic engineering.

**[0084]** According to a fifth aspect, the invention provides a plant and propagating material thereof which contain in their genome a DNA sequence of the invention or a vector as defined above.

**[0085]** In a preferred embodiment, the invention provides a genetically modified strawberry or lemon plant and propagating material derived therefrom which have a genome comprising an expression vector for over-expression or down-regulation of an endogenous strawberry or lemon plant gene counterpart of any of the DNA sequences of the invention.

**[0086]** In mammalian cells, a number of viral-based systems may be utilized. In cases where the adenovirus is used as an expression vector, a gene coding sequence(s) of the invention may be ligated into the adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will result in a variable virus capable of expressing the gene sequence(s) in infected host cells (Logan and Shark) (1984) *Proc. Natl. Acad. Sci* 81:3655-59). In addition, transcription enhancers, such as the *Rous sarcoma* virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

**[0087]** Furthermore, insect cells such as silkworm cells or larvae themselves may be used as a host. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia larvae*. The

gene coding sequence(s) of the invention may be cloned into the nonessential region of the virus, such as the polyhedrin gene, and placed under control of a polyhedrin promoter. Successful insertion of the sequence(s) will render the polyhedrin gene inactive and produce recombinant virus lacking a protein. The recombinant viruses are then used to infect *S frugiperda* cells or *Trichoplusia larvae* in which the gene sequence or sequences are expressed (Smith *et al.*) (1993) *J. Virol.* 46:584; Engelhard *et al.* (1994) *Proc. Natl. Acad. Sci.*, 91: 3224-7).

[0088] The control elements or regulatory sequences of the described systems vary in their strength and specificities and are those nontranslated regions of the vector, enhancers, promoters and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Dependent on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript® phagemid (Stratagene, La Jolla) or pSPORT (Gibco BRL) and ptrp-lac hybrids and the like may be used. Other conventionally used promoters are trc and trc promoters. The baculovirus polyhedrin promoter may be used for insect cells. As a promoter for filamentous organisms, for example, amalyse, trp C, and the like may be used. As a promoter for yeast, for example, glyceraldehyde-3-phosphate dehydrogenase may be used. As a promoter for animal cell hosts, viral promoters such as the SV40 early promoter, SV40 late promoter and the like may be used. In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as the alpha factor, alcohol oxidase and PGH may be used. For review, see Grant *et al.*, 1987 *Methods in Enzymology* 153: 516-544.

[0089] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the polypeptides of the invention may be transformed using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for one to two days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant

clumps of stable transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

**[0090]** Although the presence/absence of the marker gene expression suggests that the gene of interest is also present, its presence and expression must be confirmed. For example, if the gene sequence(s) of the invention are inserted within a marker gene sequence, recombinant cells can be identified by the absence of gene function. Alternatively, a marker gene can be placed in tandem with the gene sequence of interest, under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

**[0091]** Any number of selection systems may be used to recover the transformed cell lines. Antimetabolite, antibiotic or herbicide resistance can be used as the basis of selection; for example, dhfr which confers resistance to methotrexate (Wiger M. *et al.* (1980) *Proc. Natl. Acad. Sci.* 77: 3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin F. *et al.* (1981) *J. Mol. Biol.* 150: 1-14) and als and pat, which confer resistance to chlorsulfuron and phosphinotricin acyl transferase, respectively (Murry *supra*). Additional selectable genes have been transcribed, for example, trpB, which allows cells to utilize indole in place of tryptophan, or His D, which allows cells to utilize histinol in place of histidine (Hartman S.C. and R.C. Mulligan (1988) *Proc. Natl. Acad. Sci.* 85: 8047-51). Alternatively, one could use visible markers such as anthocyanidins,  $\beta$ -glucuronidase and its substrates, GUS, GFP and variants, and luciferase and its substrate, luciferin, which are widely used to identify transformants, but also to quantify the amount of stable protein expression attributable to a specific vector system (Rhodes C.A. *et al.* (1995) *Methods Mol. Biol.* 55: 121-131).

**[0092]** Host cells transformed with a nucleotide sequence(s) of the invention may be cultured under conditions suitable for expression and recovery of the encoded protein from cell culture. The protein produced by recombinant cells may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those skilled in the art, expression vectors containing polynucleotide sequences of the invention can be designed with signal sequences for direct secretion of the protein product through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the gene sequences of the invention to a

nucleotide sequence encoding a polypeptide domain, which will facilitate purification of soluble proteins (Kroll D.J. *et al.* (1993). *DNA Cell. Biol.* 12: 441-53).

**[0093]** According to a sixth aspect, the invention provides a method for producing aromatic and/or aliphatic esters in microorganisms, plant cells or plants comprising inserting into the genome of the microorganism or plant one or more copies of DNA sequences of the invention and feeding an alcohol and an acyl-CoA to the microorganism or plant (cell).

**[0094]** Alternatively, host cells which contain the gene sequences or polypeptides thereof according to the invention may be identified by a variety of procedures known to those skilled in the art. These procedures include, but are not limited to, DNA-DNA, DNA-RNA hybridization, amplification using probes (portions or fragments of polynucleotides), protein bioassay or immunoassay techniques which include membrane- solution- or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

**[0095]** Nucleic acid amplification-based assays involve the use of oligonucleotides or oligomers based on the gene sequences of the invention to detect transformants containing DNA or RNA encoding the polypeptides. As used herein, "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least 10 nucleotides and as many as about 60 nucleotides, preferably about 15-30 nucleotides, and more preferably about 20-25 nucleotides which can be used as a probe or a primer. Polynucleotide sequences according to the invention encoding alcohol acyl transferase polypeptides may be used to detect volatile ester (aliphatic and aromatic) formation in fruits. PCR, as described in US Patent Nos. 4,683,195 and 4,965,188, provides additional uses for oligonucleotides based on the alcohol acyl transferase sequence. Such oligomers are chemically synthesized, but they may be generated enzymatically or produced from a recombinant source. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'-3') and one with antisense (3'-5'), employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences. For example, polynucleotide sequences encoding the alcohol acyl transferase protein may be used in the hybridization or PCR assay of plant tissues, more specifically fruit tissues, to detect alcohol acyl transferase protein presence. The form of such quantitative methods may include Southern or Northern analysis, dot/slot blot or other membrane based technologies; PCR technologies such as DNA Chip, Taqman and ELISA Technologies. All of these technologies are well known in

the art and are the basis of many commercially owned diagnostic kits. The polynucleotide sequences according to the present invention herein provide the basis for assays that detect signal transduction events associated with volatile ester production, aliphatic and aromatic, hence flavour formation.

**[0096]** A variety of protocols for detecting and measuring expression of the polypeptides of the invention using either polyclonal or monoclonal antibodies specific for the protein are well known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These methods are described, among other places, in Hampton R. *et al.* (1990), Serological methods, a laboratory manual, APS Press, St Paul MN) and Maddox D.E. *et al.* (1993, *J. Exp. Med.* 158:1211).

**[0097]** A variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides, as outlined in the first and second aspects of the invention, include oligolabeling, nick translation, and end labeling of PCR amplification using a labeled nucleotide. Alternatively, the gene sequence or sequences of the invention, or any portion of it, may be cloned into a vector for the production of the mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in-vitro* by addition of an appropriate RNA polymerase, such as T7, T3, or SP6 and labeled nucleotides. A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like.

**[0098]** According to another aspect of the invention, natural, modified or recombinant polynucleotide sequences according to the invention may be ligated to a heterologous sequence to encode a fusion protein. A fusion protein may be engineered with one or more additional domains added to facilitate protein purification (i.e., a cleavage site located between the sequence of the polypeptide and the heterologous protein sequence) so that the polypeptide of the invention may be purified away from the heterologous moiety. The invention further contemplates the creation of fusion proteins, chosen from the group of sequences of the invention, degenerate variants thereof, or functional equivalents thereof, pertaining to “fruit” of strawberry and lemon, or gene sequences

with substantial homology to the sequences of the invention from other “fruit” tissues concerned with the pathway for the formation of aliphatic or aromatic esters, hence flavour. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulins and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA). The introduction of a cleavable linker such as factor XA, thrombin or enterokinase (Invitrogen, San Diego CA) between the purification domain and the polypeptide is useful to facilitate purification. One such expression vector provides for the expression of a fusion protein comprising gene sequence(s) which encode polypeptides of the invention, and contains nucleic acid encoding six histidine residues followed by thioredoxin and the enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath *et al.*, (1992) Protein Expression and Purification 3:263-281) while the enterokinase cleavage site provides a means for purifying the polypeptide from the fusion protein.

**[0099]** According to a seventh aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 27, or
- (b) a polypeptide having at least 80% homology with the amino acid sequence SEQ ID NO: 27, or
- (c) a fragment of polypeptide (a) or (b),  
which polypeptide or fragment of polypeptide has aminotransferase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0100]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 9 which encodes a polypeptide having aminotransferase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 9, or

- (c) which has at least 70% homology with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0101]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 27, or
- (b) having at least 80% homology with the amino acid sequence SEQ ID NO: 27, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has aminotransferase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

According to an eighth aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 28, or
- (b) a polypeptide having at least 90% homology with the amino acid sequence SEQ ID NO: 28, or
- (c) a fragment of polypeptide (a) or (b),  
which polypeptide or fragment of polypeptide has thiolase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0102]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 3 which encodes a polypeptide having thiolase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 3, or
- (c) which has at least 75% homology with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0103]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 28, or
- (b) having at least 90% homology with the amino acid sequence SEQ ID NO: 28, or

- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has thiolase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0104]** According to a ninth aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 29, or  
(b) a polypeptide having at least 90% homology with the amino acid sequence SEQ ID NO: 29, or  
(c) a fragment of polypeptide (a) or (b),  
which polypeptide or fragment of polypeptide has pyruvate decarboxylase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0105]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 4 which encodes a polypeptide having pyruvate decarboxylase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or  
(b) complementary to SEQ ID NO: 4, or  
(c) which has at least 75% homology with any of the sequences of (a) or (b), or  
(d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0106]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 29, or  
(b) having at least 90% homology with the amino acid sequence SEQ ID NO: 29, or  
(c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has pyruvate decarboxylase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0107]** According to a tenth aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 30, or
- (b) a polypeptide having at least 75% homology with the amino acid sequence SEQ ID NO: 30, or
- (c) a fragment of polypeptide (a) or (b),  
which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0108]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 5 which encodes a polypeptide having alcohol dehydrogenase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 5, or
- (c) which has at least 65% homology with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0109]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 30, or
- (b) having at least 75% homology with the amino acid sequence SEQ ID NO: 30, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0110]** According to an eleventh aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence selected from the group consisting of sequences SEQ ID NO: 31, 32, 33, and 34, or
- (b) a polypeptide having at least

- i) 55% homology with the amino acid sequence of a 326 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 31, or
  - ii) 75% homology with the amino acid sequence of a 278 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 32, or
  - iii) 65% homology with the amino acid sequence of a 284 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 33, or
  - iv) 80% homology with the amino acid sequence of a 188 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 34, or
- (c) a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0111]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) selected from the group consisting of sequences SEQ ID NO: 35, 36, 37 and 38, which encodes a polypeptide having alcohol dehydrogenase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 35, 36, 37 or 38, or
- (c) which has at least 55% homology, preferably at least 65% homology, with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0112]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence selected from the group consisting of sequences SEQ ID NO: 31, 32, 33 and 34, or
- (b) having at least
  - i) 55% homology with the amino acid sequence of a 326 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 31
  - ii) 75% homology with the amino acid sequence of a 278 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 32, or
  - iii) 65% homology with the amino acid sequence of a 284 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 33, or
  - iv) 80% homology with the amino acid sequence of a 188 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 34, or
- (c) being a fragment of polypeptide (a) or (b),  
which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0113]** According to a twelfth aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 39, or
- (b) a polypeptide having at least 75% homology with the amino acid sequence of a 181 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 39, or
- (c) a fragment of polypeptide (a) or (b),

which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0114]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 7 which encodes a polypeptide having alcohol dehydrogenase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 7, or
- (c) which has at least 48% homology with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0115]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 39, or
- (b) having at least 75% homology with the amino acid sequence of a 181 aa fragment from the 3' end of SEQ ID NO: 39, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0116]** According to a thirteenth aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence selected from the group consisting of sequences SEQ ID NO: 40 and 41, or
- (b) a polypeptide having at least
  - i) 55% homology with the amino acid sequence of a 176 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 40, or
  - ii) 35% homology with the amino acid sequence of a 284 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 41, or
- (c) a fragment of polypeptide (a) or (b),

which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0117]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) selected from the group consisting of sequences SEQ ID NO: 8 and 42, which encodes a polypeptide having alcohol dehydrogenase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 8 or 42, or
- (c) which has at least 20% homology, preferably at least 30% homology, with any of the sequences of (a) or (b), or
- (d) which is capable of hybridizing under stringent conditions to any of the sequences (a) - (c).

**[0118]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence selected from the group consisting of sequences SEQ ID NO: 40 and 41, or
- (b) having at least
  - i) 55% homology with the amino acid sequence of a 176 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 40, or
  - ii) 35% homology with the amino acid sequence of a 284 aa fragment from the C terminal end of the coding sequence of SEQ ID NO: 41, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has alcohol dehydrogenase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0119]** According to a further aspect, the invention provides an isolated DNA sequence encoding

- (a) a polypeptide having an amino acid sequence as shown in SEQ ID NO: 43, or

- (b) a polypeptide having at least 41% homology, preferably at least 50% and more preferably at least 80% homology, with the amino acid sequence SEQ ID NO: 43, or
- (c) a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has esterase activity and is involved in the biosynthesis pathway for aliphatic and/or aromatic ester production in fruit.

**[0120]** In particular, the invention provides an isolated DNA sequence having a nucleic acid sequence

- (a) as shown in SEQ ID NO: 44 which encodes a polypeptide having esterase activity and being involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit, or
- (b) complementary to SEQ ID NO: 44, or
- (c) which has at least 53% homology, preferably at least 70% homology, with any of the sequences of (a) or (b).

**[0121]** Further, the invention provides a purified and isolated polypeptide

- (a) having an amino acid sequence as shown in SEQ ID NO: 43, or
- (b) having at least 41% homology, preferably at least 50% and more preferably at least 80% homology, with the amino acid sequence of SEQ ID NO: 43, or
- (c) being a fragment of polypeptide (a) or (b), which polypeptide or fragment of polypeptide has esterase activity and is involved in the biosynthetic pathway for aliphatic and/or aromatic ester production in fruit.

**[0122]** Additionally, the invention provides nucleotide sequences which are suitable as primers for obtaining additional alcohol acyl transferase sequences from various fruit by cloning. These nucleotide sequences (motifs) encode:

- the amino acid sequence as set forth in SEQ ID NO: 45, or
- Leu X X X Tyr Pro X X Gly Arg, (SEQ ID NO: 46) or
- Pro Ser Arg Val X X Val Thr X Phe Leu X Lys X Leu Ile (SEQ ID NO: 47)

wherein X refers to any amino acid residue.

**[0123]** According to a fourteenth aspect, the invention further provides a method for regulating aliphatic and/or aromatic ester formation in fruit comprising inserting into the genome of a fruit-producing plant one or more copies of one or more DNA sequences as defined above encoding various enzymes involved in the biosynthetic pathway for aliphatic and/or aromatic ester formation in fruit.

**[0124]** The levels of protein may be increased, for example, by the incorporation of additional genes. The additional genes may be designed to give either the same or different spatial or temporal patterns of expression in the fruit as the target gene.

**[0125]** Further, the invention provides a genetically modified strawberry or lemon plant and propagating material derived therefrom which have a genome comprising an expression vector for over-expression or down-regulation of an endogenous strawberry or lemon plant gene counterpart of any of the DNA sequences as defined above encoding various enzymes involved in the biosynthetic pathway for aliphatic and/or aromatic ester formation in fruit.

**[0126]** Further, the invention provides a method for producing aromatic and/or aliphatic esters in microorganisms, plant cells or plants comprising inserting into the genome of the microorganism or plant one or more copies of DNA sequences of this invention encoding a polypeptide having alcohol acyl transferase activity and one or more copies of DNA sequences of this invention encoding a polypeptide having alcohol dehydrogenase activity, and feeding aldehydes and acyl-CoA to the microorganism or plant (cell).

**[0127]** In a further embodiment, the above method further comprises inserting one or more copies of DNA sequences of this invention encoding a polypeptide having pyruvate

decarboxylase activity and feeding alpha-keto acids and acyl-CoA to the microorganism or plant (cell).

**[0128]** In a still further embodiment, the above method further comprises inserting one or more copies of DNA sequences of this invention encoding a polypeptide having aminotransferase activity and feeding amino acids and acyl-CoA to the microorganism or plant (cell).

**[0129]** In a still further embodiment the above method further comprises inserting one or more copies of DNA sequences of this invention encoding a polypeptide having thiolase activity and feeding amino acids and fatty acids to the microorganism or plant (cell).

**[0130]** The DNA sequences of this invention encoding a polypeptide having esterase activity can be disrupted to increase the amount of volatile esters. Molecular strategies to accomplish this are well known in the art.

**[0131]** Strawberry and citrus are among the most popular fruits for natural flavour ingredients because of their flavour, fragrance, aroma and scent. Examples of commercially important aliphatic esters are ethyl acetate (used in artificial fruit and brandy flavours), ethyl butyrate (the primary constituent of apple aromas), isoamyl acetate (the main component of banana aroma) and ethyl butyrate (has a fruity odor, reminiscent of pineapple). Flavour usage of these esters is among the highest of all natural flavour ingredients. The invention further contemplates the use of the polynucleotide sequences according to the present invention for the industrial production of “fruit” flavours which are natural to match the odor fidelity of the natural fruit. As the chemical composition of flavour is quite complex, both the type and ratio of compounds present being the key determinant of flavour quality, the present invention further contemplates the production of novel flavours by the use of the polynucleotide sequences according to the present invention, alone or in combination, to provide novel avenues for natural flavour production in the future.

**[0132]** The invention further contemplates the generation of strawberry and lemon plants which, among other phenotypic modifications, may have one of the following characteristics:

- (a) reduced/enhanced flavour from the reduced/enhanced production of volatile esters,
- (b) increased disease resistance due to the enhanced production of volatile esters,
- (c) modified plant-insect interactions, to include increased insect resistance.

**[0133]** In a further aspect, the gene sequences of the present invention are used for the manipulation of flavour in other “fruits” and/or industrial processes. The genes/peptides of the present invention, in particular SEQ ID NO: 1 (strawberry SLE27) and SEQ ID NO: 6 (lemon CLF26), may be used in the following industrial applications, with particular reference to the manipulation of fruit- derived products, however, not exclusively limited to these:

- a) in the processed food industry as food additives to enhance the flavour of syrups, ice-creams, frozen desserts, yoghurts, confectioneries, and like products,
- b) flavouring agents for oral medications and vitamins,
- c) providing additional flavour/aroma in beverages, including alcoholic beverages,
- d) enhancing or reducing fruit flavour/aroma/fragrance/scent,
- e) enhancing the flavour/aroma of “natural products” (e.g., flower scent, however, including but not exclusively limited to flower scent),
- f) enhancing the flavour/aroma of “synthetic or artificial products”,
- g) as an alternative to the industrial synthesis of nature identical flavour/aroma substances,
- h) for the production of novel combinations of artificial flavour substances,
- i) as antibacterial or as antifungal agents,
- j) fragrance /perfumes in cosmetics, creams, sun-protectant products, hair conditioners, lengthening agent and fixative in perfumes, suspension aid for aluminium salts in antiperspirants pharmaceuticals, cleaning products, personal care products and animal care products,
- k) disinfectant additives,
- l) as degreasing solvents for electronics, etc.,
- m) insect pheromones,
- n) dye carrier, solvents, insect repellent, miticide, scabicide, plasticizer, deodorants.

**[0134]** An example by way of illustration of the industrial importance of volatile esters (aromatic and aliphatic) is the ester methyl anthranilate. Methyl anthranilate, a methyl ester of o-aminobenzoic acid, apart from being a very important perfumery, aroma, scent ester (it imparts a peculiar aroma known as “foxy” to grapes of the North American species *Vitis Labrusca*), is known to serve as a pheromone in various insects. In results of investigations aimed at testing the

attractiveness of methyl anthranilate for the soybean beetle *Anomala rufocurea*, Motschulsky suggested the potential use of methyl anthranilate as a lure for mass trapping, as well as for monitoring (Imai *et al.*, Applied Entomology and Zoology, 32: 1, 45 - 48, 1997). In addition methyl, anthranilate acts as a bird repellent. According to the invention, we propose the use of the SEQ ID NO: 2 for the production of methyl anthranilate, for a number of applied applications.

**[0135]** The present invention also relates to antibodies which specifically bind to the polypeptides as claimed in the invention, compositions comprising substantially purified polypeptides, fragments thereof, agonists or, alternatively antagonists, and methods of producing polypeptides. In bacterial/prokaryote systems, a number of expression vectors may be selected depending on the use of the polypeptide(s) according to the invention. For example, when large quantities of the polypeptide are needed for the induction of the antibody, vectors which drive high levels of expression of fusion proteins that are readily purified are desirable. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors, such as Bluescript® (Stratagene), in which the gene sequence(s) according to the invention can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase so that the hybrid protein is produced; pPIN vectors (Van Heekle and Schuster (1989) *J. Biol. Chem.* 264: 503-5509); and the like. pGEX vectors (Promega, Madison WI) may also be used to express foreign polypeptides as fusion proteins with glutathione-S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by absorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems are designed to include heparin or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

**[0136]** In addition to recombinant production, the amino acid sequences of the polypeptides, or any parts thereof, may be altered by direct synthesis or by genetic means known by those skilled in the art, and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide. Fragments of the polypeptides according to the invention may be produced by direct peptide synthesis using solid phase techniques (C.F. Stewart *et al.* (1969) Solid Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, Merrifield J (1963) *J. Am. Chem. Soc.* 85:2149-2154). *In vitro* protein synthesis may be performed using manual

techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizers (Perkin Elmer, Foster City CA) in accordance with the instructions provided by the manufacturer. Various fragments of each of the polypeptides may be chemically synthesized separately and combined using chemical methods to produce full-length molecules.

**[0137]** The invention further contemplates a diagnostic assay using antibodies raised to each of the polypeptides of the present invention, with or without modification. For example, although not exclusively limited, alcohol acyl transferase antibodies raised to the polypeptides as outlined in the 6-8th embodiments are useful for the detection of flavour-forming esters in various fruits. Diagnostic assays for alcohol acyl transferase include methods utilizing the antibody and a label to detect the alcohol acyl transferase protein in various fruit extracts. Frequently, the polypeptides and antibodies are labeled by joining them, either covalently or noncovalently, with a reporter molecule. A variety of reporter molecules are known and several of them have been previously described (cf. reporter genes).

**[0138]** A variety of protocols for measuring the polypeptide, using either polyclonal or monoclonal antibodies specific for the respective protein, are known in the art. Polynucleotide sequence(s) of the invention, SEQ ID NO: 1 strawberry (SLE27), SEQ ID NO: 2, lemon alcohol acyl transferase proteins (CLF26), or any part thereof, may be used for diagnostic purposes to detect and quantify gene expression in various tissues (e.g., leaves, stems and in particular fruits (climacteric and nonclimacteric)), hence volatile ester (aliphatic and aromatic) formation, hence flavour at various stages of plant maturation. The diagnostic assay is useful to distinguish between the absence, presence and excess expression of the alcohol acyl transferase proteins and to monitor expression levels during development.

**[0139]** The following examples are offered by way of illustration and not by limitation.

#### **Example 1: Method for the detection of volatiles**

**[0140]** The composition of the headspace of the strawberries was analyzed using Solid Phase Micro-extraction (SPME) for trapping and concentrating the emitted volatiles. The fibers used were 100  $\mu$ m Polydimethyl siloxane (PDMS) supplied by Supelco. Analysis was performed on a

Fisons GC 8000 system, coupled to the Fisons MD800 mass spectrometer. The gas chromatograph was temperature programmed, with a plateau at 80°C for 2 min., followed by a ramp of 8°C/min to 250°C for 5 min. The injector port was operated at 250°C for thermal desorption during 60 sec. of the SPME fibers, using the deactivated standard 0.7 ml split/splitless injection port liner with zero split. The column used was an HP-5 with a length of 50 m and internal diameter of 0.32 mm and a film thickness of 1.03 µm, and operated with helium at a pressure of 38kPa. Eluated components were identified by their mass spectrum, using the Fisons Masslab software and the NIST library. The identity of the components was verified where possible by using known substances as reference (most of them obtained from Aldrich).

[0141] Headspace analyses were performed both on the plant and in the laboratory using detached fruits. The fruits were put onto new clean aluminium foil and covered with a glass funnel closed with aluminium foil. The SPME fiber was inserted in the funnel through the foil closing, and the fiber was exposed to the air in the funnel during 30 min. Figure 2 shows a typical chromatogram obtained after headspace measurement of red ripe fruit of strawberry grown in the greenhouse. The importance of esters to the overall volatile emission from the ripe strawberry fruit can be clearly observed.

## **Example 2: Construction of a strawberry red fruit stage and lemon young fruit peel cDNA library, mass excision and random sequencing**

### **2.1 Messenger RNA isolation and cDNA libraries construction**

[0142] Total RNA was isolated from strawberry red fruit stage of development using the method described by Manning K. (Analytical Biochemistry 195, 45-50 (1991)). The cultivar used was *Fragaria X ananassa* Duch. cv. *Elsanta*. Plant material for the isolation of RNA from lemon (citrus Limon cv. *Meyer*) was the green peel of a small fruit (1.2 x 2 cm) of the cultivar. Isolation of RNA from lemon was done using the same method as described for strawberry. The cDNA libraries for both strawberry and lemon were produced as a custom service by (Stratagene) in the lambda zap vector. Messenger RNA was isolated from total RNA using the polyA<sup>+</sup> isolation kit (Pharmacia).

## 2.2 Mass excision and random sequencing

[0143] The ExAssist™/SOLR™ system (Stratagene) was used for mass excision of the pBluescript SK(-) phagemid. The excision was done according to the manufacturer's instructions using  $20 \times 10^3$  pfu from the nonamplified library for each excision. High-quality plasmid DNA from randomly picked colonies was extracted using the QIAGEN BioROBOT 9600. Colonies were grown overnight in 3ml Luria Broth medium (10g/l tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) supplemented with 100 mg/litre Ampicillin, centrifuged at 3000 RPM for 10 min. and the pellet was used directly for plasmid DNA isolation by the robot. Each DNA isolation round consisted of 96 cultures.

[0144] Insert size was estimated by agarose gel electrophoresis after restriction enzyme digestion of the pBlueScript (SK-) vector with EcoRI and XhoI. Inserts with lengths above 500 bp were used for sequencing. Plasmid DNA from the selected samples was used for polymerase chain reaction (PCR) sequencing reactions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and the MJ Research PTC-200 DNA Engine™ thermal cycler. The T3 and T7 universal primers were used for sequencing from the 5' and 3' ends respectively. PCR program was according to the Dye Terminator manufacture's protocol (ABI PRISM). The ABI 373, 370A and 310 sequencers (Applied Biosystems) were used. Sequences were edited manually to remove vector and nonreliable sequences and submitted to the Blast homology search program using BlastX and BlastN (Altschul *et al. J. Mol. Biol.* 215, 403 - 410, 1990) provided by the National Centre for Biotechnological Information on the world wide web. The strawberry and lemon sequencing data received from the Blast search was transferred using a self-designed macro program to a database made using the software Access (Microsoft). Sequences with a BlastX score above 80 were considered as having a significant homology (Pearson, *Curr. Opinion. Struct. Biol.*, 1, 321 - 326, 1991) and they were used for data interpretation in the Access and Excel programs (Microsoft). The cDNA clones: SLE27 (SEQ ID NO: 1), CLF26 (SEQ ID NO: 2), SLF96 (SEQ ID NO: 9), SLG150 (SEQ ID NO: 3), SLH51 (SEQ ID NO: 4), SLB39 (SEQ ID NO: 5), SLF193 (SEQ ID NO: 35), SLF122 (SEQ ID NO: 36), SLD194 (SEQ ID NO: 37), SLF17 (SEQ ID NO: 38), SLF138 (SEQ ID NO: 7), SLG16 (SEQ ID NO: 8), SLG144 (SEQ ID NO: 42) were selected for further analysis.

### **Example 3: Characterization of selected cDNAs**

#### **3.1 Sequencing the entire length cDNAs**

[0145] Plasmid DNA from the selected cDNAs was used for PCR sequencing reactions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and the MJ Research PTC-200 DNA Engine™ thermal cycler. Each individual selected cDNA was sequenced completely on both strands. DNA and protein alignment “contigs” were performed using the GeneWorks software (IntelliGenetics, Oxford).

#### **3.2 RNA isolation from different tissues and organs of strawberry and lemon.**

[0146] RNA isolation from both strawberry and lemon tissues was done as described for the preparation of total RNA for the construction of the cDNA libraries (Manning K. *Analytical Biochemistry* 195, 45-50 (1991)). For both species green house grown plant material was used. From strawberry, the different tissues/organs used for RNA isolations were: leaf, root, petiole, flower, green fruit, white fruit, turning fruit, red fruit, red fruit without seeds, seeds and overripe fruit. For lemon, the different tissues/organs used were: root, leaf, flower bud, albedo, green fruit peel (1.2 x 2 cm), green fruit peel (2 x 3 cm), green fruit peel (3.5 x 6 cm), green fruit peel (6 x 8 cm), yellow fruit peel (2 weeks fruit detached).

#### **3.3 Expression analysis using Northern blots**

[0147] Ten µg of each RNA sample was denatured by glyoxal (1.5M) prior to electrophoresis on a 1.4% agarose gel in 15mM sodium phosphate buffer pH=6.5 (without Ethidium bromide). Equal loading of RNA was verified by visualizing equal amounts of RNA from each sample on a 1% TAE buffer (1x : 0.04M Tris-acetate / 0.001M ethylenediaminetetra-acetate (EDTA)) agarose gel prior to loading. Blotting was done using Hybond N<sup>+</sup> membrane (Amersham) in 25 mM sodium phosphate buffer pH = 6.5 overnight.

[0148] After fixation (2h 800c), blots were hybridized as described by Angenent *et al.* 1992 (*Plant Cell* 4, 983-993). The hybridization probes were made by random labeling oligonucleotide priming (Feinberg and Vogelstein, *Anal. Biochem.* 137, 266-267, 1984) of the entire

cDNAs. The hybridized membranes were autoradiographed at -70°C with intensifying screens. In Figure 3, the expression pattern of strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1) is demonstrated by a Northern blot loaded with total RNA extracted from 10 different plant tissues and/or organs from strawberry. The expression pattern of strawberry alcohol acyl transferase is in perfect match with the accumulation of volatile esters in the strawberry plant. The gene SLE27, (SEQ ID NO: 1) is expressed at its lowest level in the white fruit, gradually increasing as fruit develops, reaching its highest levels in the turning stage and the red stage when the volatile ester compounds can be detected at their maximum. In other strawberry plant tissues/organs which do not produce those volatile fruity esters, the expression of the gene cannot be detected even when the film is exposed to a longer period after hybridization.

**[0149]** The expression of the lemon alcohol acyl transferase (CLF26 SEQ ID NO: 2) seems to be correlated with the accumulation of volatile monoterpene compounds in the lemon tree tissues/organs (Figure 4). These compounds can be converted by this enzyme to their ester derivatives as a result of alcohol acyl transferase enzyme activity. The gene is more highly expressed in the flower bud. Analysis by Gas chromatograph (GCMS) shows high concentrations of terpene compounds produced in the flower bud such as limonene. Expression analysis of monoterpene cyclases (genes directly responsible for the formation of monoterpenes) isolated from lemon thus shows a similar pattern of expression to the lemon alcohol acyl transferase (CLF26, SEQ ID NO: 2). Expression of the gene was also strongly detected in the early stages of fruit development and in the flower bud, while no expression could be detected in the root and albedo tissues. This data provides evidence for a link between the presence of terpene compounds, mainly monoterpenes and the lemon alcohol acyl transferase (CLF26, SEQ ID NO: 2).

**[0150]** The expression of the other three genes part of the metabolic pathway described in this invention for the formation of volatile ester compounds, in particular aliphatic esters in fruits, is shown in Figure 5. Thiolase (SLG150, SEQ ID NO: 3), alcohol dehydrogenase (SLB39, SEQ ID NO: 5) and pyruvate decarboxylase (SLH51, SEQ ID NO: 4) gene expression was monitored at different organs or tissues of the strawberry plant. The data shown provides evidence that thiolase (SLG150, SEQ ID NO: 3), alcohol dehydrogenase (SLB39, SEQ ID NO: 5) and pyruvate decarboxylase (SLH51, SEQ ID NO: 4) are involved in ripening processes such as the production

of strawberry flavour and aroma compounds. Clones SLF193 (SEQ ID NO: 35), SLF122 (SEQ ID NO: 36), SLD194 (SEQ ID NO: 37), SLF17 (SEQ ID NO: 38), SLF138 (SEQ ID NO: 7), SLG16 (SEQ ID NO: 8), SLG144 (SEQ ID NO: 42) and SLF96 (SEQ ID NO: 9) were also tested for the expression and they were all appearing to have upregulated expression in the ripening fruit (data not shown).

### **3.4 Coordinate expression of genes in the biosynthetic pathway leading to aromatic and aliphatic ester formation**

[0151] The coordinate expression pattern of genes of the biosynthetic pathway leading to aromatic and aliphatic ester formation was simultaneously monitored using arrays of cDNAs (Ermolaeva *et al.*, 1998, *Nature Genetics*, Vol, (20), 19-23). The method allows one to simultaneously quantitatively and statistically analyze the expression profiles of genes pertaining to a particular metabolic pathway, such as the pathway leading to aliphatic and aromatic ester formation, flavour, fragrance, aroma and scent. The experimental and hybridization conditions followed were according to Schena *et al.*, (PNAS, 93: 10614 - 10619). The expression levels derived from four images were statistically analyzed with an analysis of variance modeling the factors dye, origin, and spot levels on a logarithmic scale giving the significant ratios of expression between the spots of origin (e.g., green versus red). By way of illustration, the expression profiles of SEQ ID NO: 1, SEQ ID NO: 9, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 8 analyzed in the green versus red and turning versus red stages of fruit development, are outlined in Figure 10. The coordinated expression pattern of the genes during strawberry fruit development is in perfect match with the accumulation of aromatic and aliphatic volatile ester compounds during strawberry fruit development. In addition, alcohol acyl transferase enzyme activity was detected by Perez *et al.*, (J. Agric. Food Chem. 1996, 44, 3286-3290) to be elevated dramatically between the white and the turning stages of strawberry fruit development. This correlates well with a dramatic increase in expression of the genes of the biosynthetic pathway as shown in Figure 10. A supporting detail is the fact that volatile ester compounds can be detected in their maximum when the fruit is completely red and this is probably a result of gene activation at earlier stages between the white and turning stages of development.

### 3.5 DNA isolation from strawberry leaf tissue

[0152] Genomic DNA was isolated from greenhouse-grown strawberry plants (cv. *Elsanta*). Young folded leaves kept 2 days in water in the dark were used for the isolation. One gram of leaf material was ground in liquid nitrogen and immediately added to 7 ml DNA extraction buffer (100 mM Tris-Cl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2%  $\beta$ -mercaptoethanol). After incubation for 30 min. at 60°C, one volume of chloroform:isoamyl alcohol (24:1) was added to the tubes. The samples were then centrifuged for 10 min. at 4000 rpm and the aqueous phases were transferred to 4.5 ml isopropanol and incubated for 30 min. at -20°C. Nucleic acids were spun down at 4000 rpm for 12 min., washed with 70% ethanol and air dried. Pellets were dissolved in 3 ml TE and 0.25 volumes of NaCl (5M) and 0.1 volume absolute ethanol were added. Samples were centrifuged for 10 min. at 4000 rpm and supernatants were transferred to 4.5 ml isopropanol for precipitation. Pellets were washed with 70% ethanol, dried and dissolved in 0.5 ml TE buffer (10mM Tris-Cl, 1mM EDTA pH = 8.0). RNA was selectively precipitated by the addition of 0.6 volumes of 8M lithium chloride. The RNA was pelleted by centrifugation for 10 min. at 4000 rpm and the DNA was precipitated again with the addition of 0.6 volume isopropanol. Pellets were washed with 70% ethanol, dried and dissolved in 0.5 ml sterile water. The amount of DNA was determined by comparing to known amounts of phage lambda DNA.

### 3.6 Southern blot analysis for strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1)

[0153] Aliquots of 2  $\mu$ g strawberry genomic DNA were digested for 16h with the restriction enzymes: HindIII, XbaI, XhoI and EcoRI and separated on a 0.7% TAE (0.04M Tris-acetate pH = 8.0 and 1mM EDTA pH = 8.0) agarose gel. The DNA was then denatured in 0.4M NaOH for 30 min. and then transferred to a Hybond N+ membrane in 0.4M NaOH. Blot hybridization and probe preparation were done as described previously for Northern blots. After hybridization, the blot was washed first under low stringency conditions (52°C with 2 times half an hour 2xSSC/0.1% SDS) and, after observation of the results by autoradiography of the film they were washed further under stringent conditions (65°C with 2 times half an hour 0.1xSSC/0.1% SDS) and autoradiographed again.

**[0154]** In order to investigate whether the different esters formed in the fruit are a result of an activity of one single enzyme or a multigene family, a Southern blot was performed using strawberry genomic DNA and the full-length SLE27 cDNA (SEQ ID NO: 1) which served as a probe (Figure 6). Since the cultivar used for the investigation is an octaploid, the interpretation of the results is more difficult than that for a diploid plant. However, the results obtained suggest the possible existence of another homolog of strawberry alcohol acyl transferase. This additional copy might arise from one of the parental lines with a similar function to the one isolated, or may represent another homolog with different function or substrate specificity to SLE27 (SEQ ID NO: 1).

### **3.7 Alcohol acyl transferase sequences from other fruit**

**[0155]** Additional alcohol acyl transferase sequences from melon, strawberry vesca, banana, apple, lemon and mango were obtained using degenerate primers synthesized to the motif SEQ ID NO: 45 using methods well known in the art such as polymerase chain reaction (PCR) and reverse genetic approaches such as RT-PCR. Such techniques are described in Sambrook *et al.*, 1989, Molecular cloning a laboratory manual, Cold Spring Harbor Press, Plain View, NY and Ausubel, F.M. *et al.* (1989), Current protocols in molecular biology, John Wiley and Sons, New York, NY. The esterase gene was cloned using PCR techniques using the following degenerate primer: 5'-GG (T/A) TGGGG I (T/G) CTA(T/C) TCTTGC-3' (SEQ ID NO: 48) and reverse genetic approaches such as RT-PCR. Such techniques are described in Sambrook *et al.* (1989). Molecular cloning a laboratory manual, Cold Spring Harbor Press, Plain View, NY and Ausubel, F.M. *et al.* (1989), Current protocols in molecular biology, John Wiley and Sons, New York, NY.

### **Example 4**

#### **Strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 6) protein analysis**

##### **4.1 Cloning to the expression vector pRSETB**

**[0156]** The *E. coli* expression vector pRSETB (Invitrogen) was used for expression studies of strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1). This pRSETB vector contains the T7 promoter which can be induced by isopropyl-beta-D- thiogalactopyranoside (IPTG) and, therefore, by inserting the desired gene downstream of this promoter, the gene can be expressed

in *E. coli*. In addition, DNA inserts were positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes (in 5' to 3' order from the N-terminal to the C-terminal), an ATG translation initiation codon, a series of six Histidine residues that functions as a metal binding domain in the translated protein, the Anti-Xpress epitope, and the enterokinase cleavage recognition sequence.

[0157] The original pRSETB was primarily used for the insertion of the gene encoding the Green Fluorescent Protein (GFP). The GFP gene was fused to the pRSETB vector using the BamHI and HindIII restriction sites located at the multiple cloning site (MCS), as can be seen in Figure 7. This construct for the expression of GFP served as control for the experiments together with the empty pRSETB vector.

[0158] Cloning the GFP gene to the pRSETB vector inserted an additional SalI restriction site at the 3' end of the GFP gene and, together with the BamHI site located at the 5' end of the GFP gene, served as sites for the cloning of the strawberry alcohol acyl transferase gene (SLE27, SEQ ID NO: 1). The BamHI and SalI sites were introduced to the 5' and 3' ends/positions respectively of the strawberry alcohol acyl transferase coding sequence by the use of PCR. The 452 amino acid open reading frame of the SLE27 (SEQ ID NO: 1) clone was amplified with the pfu DNA polymerase (Stratagene) and primers AAP165 (5'-CGGATCCGGAGAAAATTGAGGTCAG) (SEQ ID NO: 49) and AAP166 (5'-CGTCGACCATTTGCACGAGCCACATAATC) (SEQ ID NO: 50) according to the manufacturer's instructions. The PCR product was cloned into PCR-script vector (Stratagene), cut out with BamHI and SalI and further inserted (as a translation fusion) into the corresponding restriction sites in the pRSETB vector.

#### **4.2 Bacterial expression and partial purification using the His tag columns**

[0159] The pRSETB vector harboring the strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1) was used to transform *E. coli* strain BL21 Gold DE3 (Stratagene) as described by the manufacturer. For bacterial expression, typically 1 ml of overnight liquid culture grown at 37°C in Luria Broth (LB) medium (10g/l tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) supplemented with 100 mg/liter Ampicillin was diluted 50 times in the same medium and grown until the OD<sub>600</sub> reached 0.4 (usually at 37°C except when different temperatures for growth during induction were examined).

At this stage, Isopropylthio-beta-D-galactoside (IPTG) was added to a final concentration of 1mM in order to induce expression. After one and a half hours of induction, the cells were harvested by centrifugation at 4000 x g for 15 min. A pellet and a sample from the supernatant were kept for SDS gel analysis. The cells were further processed as described by the Ni-NTA Spin Column's manufacturer (QIAGEN) for protein purification under native conditions.

### **4.3. Recombinant protein detection using Western blot**

**[0160]** The supernatant (S), pellet (P) and eluate from the Ni-NTA columns (E) samples were diluted with 2x SDS-sample buffer (standard Laemmli, pH 6.8 Tris/glycine/SDS/glycerol + 50mM Dithiothreitol(DTT)). The samples were heated for 5-10 min. at 95°C, followed by a short spin of 30 seconds, full speed. From the samples, 10µl was loaded on 10% SDS-gel (mini-gel, 1mm, 7x9cm). The gels were run with standard buffer (25mM Tris 192mM, glycine 0.1% SDS, pH 8.3) at 100-200V, 50-30mA, for about 3hr with 16°C water cooling. After complete run, gels were used for Coomassie Brilliant Blue (CBB) stain or Western blot. For CBB staining, the gel was fixed during 1 hour in 40% Methanol, 10% Acetic Acid in water, then incubated overnight with CBB staining solution (0.05% CBB-250 in 40% Methanol, 10% Acetic acid in water) and destained by repeated washing in 25% Methanol, 10% Acetic acid in water. Western blot was onto Polyvinyl difluoride (PVDF), (Immobilon P., Amersham) prewetted with Methanol. Transfer buffer was Towbin buffer plus 0.1% SDS (25mM Tris, 192mM Glycine, 20% 0.1% SDS, pH 8.3). Transfer was submarine (Novex blot unit, plate electrodes, 3cm distance) at 200mA overnight plus 2hr. at 400mA, cooled in ice-water with cooling block. Transfer was checked by using prestained markers as a reference.

**[0161]** After transfer, the blot was washed in Tris buffer saline TBS (20mM Tris-HCl pH7.5/ 150mM NaCl)- Tween20 (0.02%), blocked with 2% fat-free milk in TBS-Tween20, then washed with TBS-Tween 20. The first Antibody was anti-Xpress (Invitrogen) 1/1000 in 2% BSA TBS-Tween for 3hr. incubation at room temperature, then washed 4x with TBS-Tween 20. Second Antibody is GAR-AP (Boehringer) 1/3000 in 2% BSA TBS-Tween (TBS + 0.05% Tween - 20) for 1-2hr. incubation at room temprature, then 4x washed with TBS-Tween 20. Final wash was in water, then in Alkaline phosphatase-buffer (100mM Tris pH9.2, 1mM MgCl<sub>2</sub>). The staining reaction was

with nitroblue tetrazolium/5-bromo-4-chloro-3-indol phosphate/alkaline phosphatase-buffer (45  $\mu$ l nitroblue tetrazolium 30mg/ml in 70% dimethylformamide, 33  $\mu$ l 5-bromo-4-chloro-3-indol phosphate 15mg/ml in dimethylformamide in 10ml of alkaline phosphatase buffer). The staining reaction was stopped by rinsing with water. Western blotting was used in order to determine the amount of the recombinant SLE27 (SEQ ID NO: 6) protein in the eluate after the Ni-NTA column purification compared to the pellet and supernatant before loading on the column. Figure 9 shows a western analysis of the pellet supernatant and eluate after partial purification on the Ni-NTA column. Four samples were loaded from each pellet, supernatant and eluate originating from four different growth and induction of the *E. coli* cells. A commercial antibody recognizing an epitope peptide fused to the N-terminus of the SLE27 recombinant protein showed relatively high expression of SLE27 (SEQ ID NO: 6) in the eluate from the Ni-NTA column. The molecular weight of the recombinant SLE27 (SEQ ID NO: 6) recombinant protein was 54 kDa. The enzyme exhibited a broad pH range (pH optimum around 8.3). As a control for the experiment, the Green Fluorescent Protein (GFP) was partially purified in the same method as done for SLE27 (SEQ ID NO: 6). No band was detectable at the level of the SLE27 (SEQ ID NO: 6) recombinant protein in the GFP lane.

#### **4.4 Method for establishing alcohol acyl transferase activity of the strawberry SLE27 (SEQ ID NO: 6)-encoded protein**

[0162] The alcohol acyl transferase activity of the strawberry cDNA SLE27 (SEQ ID NO: 1)- encoded protein was established by analyzing the volatile esters produced from acyl-CoA and different alcohols. In a 1.8 ml glass vial with a small magnetic stirring bar, 325  $\mu$ l of buffer (50 mM Tris/HCl pH 8.0, containing 1 mM dithiothreitol), 25  $\mu$ l of protein extract and 25  $\mu$ l of acyl-CoA (4 mM in buffer) were subsequently pipetted. The enzyme reaction was started by the addition of 25  $\mu$ l of alcohol solution (160 mM in buffer). The vial was quickly closed with a snap cap and placed in a waterbath (at 35°C) with a stirring device. After 15 min. enzyme reaction with continuous stirring, the vial was opened, 0.29 g of solid  $\text{CaCl}_2$  was added (final concentration 5M) and the vial was quickly closed again with an open top crimp cap. The high  $\text{CaCl}_2$  concentration was used to stop further enzymatic reactions and to drive the volatiles into the headspace. After addition of  $\text{CaCl}_2$ , the mixture was incubated again at 35°C while stirring. The volatile components released into the

headspace were trapped using solid phase micro-extraction (SPME; Supelco Inc., Bellefonte, PA, USA), exposing the fused silica fiber coated with 100 nm polydimethylsiloxane (PDMS; Supelco Inc.) to the headspace for 15 min. The presence of esters in the alcohol stock solutions and the spontaneous formation of esters were checked by adding 25  $\mu$ l of buffer instead of protein extract into the reaction vial.

**[0163]** The volatiles trapped by SPME were separated and identified by gas chromatography (GC 8000 Series; Interscience B.V., Breda, The Netherlands) coupled to mass spectrometry (MD-800, Fisons Instruments, Interscience) as described by *Verhoeven et al.* (1997) (*Chromatographia* Vol. 46, No 1,2). Compounds were thermally desorbed (250°C) from the SPME-fiber for 1 min. Separation of compounds was on a capillary HP-5 column (50 m x 0.32 mm, film thickness 1.05  $\mu$ m; Hewlett Packard) with He (37 kPa) as a carrier gas. The oven temperature was programmed at 2 min. at 80°C, then increased at 8°C min<sup>-1</sup> to 250°C, and finally 5 min at 250°C. Mass spectra of eluting compounds were generated at 70eV each second. The MD 800 Masslab software (Interscience) was used to record mass spectra and to calculate peak areas. Volatile compounds were identified by screening the NIST library for comparable mass spectra and by comparison with authentic reference compounds.

#### **4.5 Substrates used by the strawberry alcohol acyl transferase-encoded enzyme (SLE 27: SEQ ID NO: 6)**

**[0164]** The histidine-released protein eluate from *E. coli* expressing the strawberry alcohol acyl transferase gene (SLE27, SEQ ID NO: 1) was incubated with both acetyl-CoA and 1-butanol. The chromatograms obtained by SPME of volatiles released into the headspace indicated a clear peak of acetic acid 1- butyl ester (Figure 8B). This ester was not detected when the protein (Figure 8C), alcohol substrate (Figure 8D) and acetyl CoA were absent (Figure 8E). Moreover, when the SLE27 (SEQ ID NO: 6)-encoded protein eluate in the assay was replaced by an equal amount of histidine-released eluate from lysate of *E. coli* expressing only the histidine-tag (i.e., the same vector without the SLE27 (SEQ ID NO: 1 gene)), the ester could not be detected either (Figure 8G). To investigate which substrates can be used by the SLE27(SEQ ID NO: 6)-encoded protein to produce volatile esters with acetyl-CoA, the alcohol species in the assay was varied. Table 1 shows all substrates

tested and the species that can be used by the SLE27-encoded enzyme (SEQ ID NO: 6). Extensive *in vitro* enzyme assays using both GC-MS and radio-GC revealed that a comprehensive array of acyl-CoAs and alcohols (short and long chain with even and uneven carbon number, aromatic, cyclic and aliphatic, branched and unbranched, saturated and unsaturated) can serve as substrates for the SLE27-encoded enzyme (SEQ ID NO: 6) *in vivo* (Table 1 & 2).

**[0165]** The alcohol acyl transferase activity of the the SLE27-encoded enzyme (SEQ ID NO: 6: *SAAT*) encoded protein was established in two ways: (1) For the activity with different alcohols and acetyl-CoA, ( $^{14}\text{C}$ )-acetyl-CoA was used as a substrate and the formation of (radio-labeled) products analyzed using radio-gas chromatography and quantified using liquid-scintillation counting. Radio-GLC was performed essentially as described previously (Bouwmeester, H.J. (1999) *Plant Physiol.* 121: 173-180 (1999)). Temperature programming was as follows: 70°C for 10 min., 10 min<sup>-1</sup> to 270°C and a final time of 10 min. For quantification of the enzyme activity, 1.4 µg SAAT protein was diluted to 100 µl with 50 mM Tris-buffer (pH 8.3), to which ( $^{14}\text{C}$ )-acetyl-CoA (0.1 mM at 0.1 Ci.mol<sup>-1</sup> in routine assays) and alcohol (20 mM in routine assays, 2 µl of hexane stock) were added. After incubation for 30 min. at 30°C, the assays were cooled on ice for 15 min. and 700 µl of hexane were added. Assays were vortexed and centrifuged, and a 600-µl portion of the hexane phase was removed for liquid scintillation counting. The ratio of hexane soluble radioactivity (esters) to the total radioactivity added as acetyl-CoA was used to calculate product formation. (2) For the utility of different acyl-CoAs, assays containing 325 µl of buffer (50 mM Tris/HCl pH 8.0, containing 1 mM dithiothreitol), 25 µl of protein eluate and 25 µl of acyl-CoA (4 mM in buffer) were stirred in a glass vial. The enzyme reaction was started by the addition of 25 µl of alcohol (160 mM in buffer). After 15 min. incubation with continuous stirring (35°C), solid CaCl<sub>2</sub> was added (final concentration 5 M). Volatiles released into the vial headspace (at 35°C with stirring) were subsequently trapped for 15 min. by exposing a fused silica fiber coated with 100 µm polydimethylsiloxane (PDMS) to the headspace for 30 min. The SPME-trapped volatiles were analyzed by GC-MS, as described previously (Verhoeven et al., (1997). *Chromatographia* 46, 63-66 ).

**[0166]** **Table 1.** Substrate specificity of the SLE27 (SAAT), SEQ ID NO: 6 recombinant enzyme towards different types of alcohols. Comparison of esterification activity with different

alcohols (20 mM), and using ( $^{14}\text{C}$ )acetyl-CoA (0.1 mM) as acyl donor. Activity (mean  $\pm$  SD,  $n = 2$ ) is expressed as nmol product formed per hour per  $\mu\text{g}$  enzyme. <sup>a</sup>- the ester product was not reported to occur in commercial strawberry varieties (data from Honkanen and Hirvi. 1990). <sup>b</sup>- no detectable activity. n.d. - not described. Ester properties are from Arctander, (1969).

Table 1

Alcohol	Carbon no.	Activity	Ester formed	Ester properties
methanol	C1:0	0.43 $\pm$ 0.10	methyl acetate	ethereal, sweet
ethanol	C2:0	0.41 $\pm$ 0.07	ethyl acetate	pineapple, ethereal
1-propanol	C3:0	2.60 $\pm$ 0.26	1-propyl acetate	powerful celery odor
2-propanol	C3:0	1.30 $\pm$ 0.15	2-propyl acetate	ethereal, sweet, banana, fruity
1-butanol	C4:0	2.29 $\pm$ 0.25	1-butyl acetate	fruity, diffusive
2-butanol	C4:0	3.11 $\pm$ 0.02	2-butyl acetate	n.d.
3-methyl-1-butanol	C5:0	3.68 $\pm$ 0.25	3-methyl-1-butyl acetate	fruity, banana, sweet
1-hexanol	C6:0	8.44 $\pm$ 0.37	1-hexyl acetate	apple, cherry, pear, floral
<i>cis</i> -2-hexen-1-ol	C6:1	6.05 $\pm$ 0.55	<i>cis</i> -2-hexenyl acetate <sup>a</sup>	n.d.
<i>cis</i> -3-hexen-1-ol	C6:1	4.06 $\pm$ 0.14	<i>cis</i> -3-hexenyl acetate	green, sharp-fruity
<i>trans</i> -2-hexen-1-ol	C6:1	9.20 $\pm$ 0.65	<i>trans</i> -2-hexenyl acetate	fresh-green, sweet, fruity
<i>trans</i> -3-hexen-1-ol	C6:1	1.25 $\pm$ 0.07	<i>trans</i> -3-hexenyl acetate <sup>a</sup>	n.d.
1-heptanol	C7:0	14.89 $\pm$ 4.12	heptyl acetate <sup>a</sup>	sweet, apricot like
1-octanol	C8:0	16.36 $\pm$ 2.69	1-octyl acetate	fruity, floral, jasmine, herba-
1-nonanol	C9:0	14.00 $\pm$ 0.11	1-nonyl acetate <sup>a</sup>	floral, fruity (mushroom-
1-decanol	C10:0	7.79 $\pm$ 0.10	1-decyl acetate	floral, orange-rose
furfuryl alcohol	C5:0	0.72 $\pm$ 0.06	furfuryl acetate <sup>a</sup>	fruity, banana
benzyl alcohol	C7:0	0.68 $\pm$ 0.04	benzyl acetate	sweet, floral, fruity, fresh
2-phenylethyl alcohol	C8:0	1.58 $\pm$ 0.12	2-phenylethyl acetate	floral, rose, honey like under-
linalool	C10:0	<sup>b</sup>	linalyl acetate <sup>a</sup>	floral-woody odor with faint

Table 2.

[0167] Acceptance of different types of acyl-CoAs as co-substrates by the SLE27 (SAAT), SEQ ID NO: 6 recombinant enzyme. Esters formed after 15 min. reaction of SAAT protein with the substrates indicated were trapped using headspace-SPME and GC-MS. <sup>a</sup>- the ester product was not reported to occur in commercial strawberries (data from Honkanen and Hirvi. 1990 *Dev. Food Sci. Amsterdam : Elsevier Scientific Publications*, 125-193.). “n.d.” - not described. Ester properties are from Arctander, *S. Perfume and Flavor Chemicals* (Steffen Arctander's Publications, Las Vegas, NV, (1969).

Table 2:

AcylCoAs (Carbon no.)	Alcohol	Ester formed	Ester properties
n-propionyl CoA (C3:0)	1-butanol	1-butyl propanoate <sup>a</sup>	ethereal, banana
n-butyryl CoA (C4:0)	1-propanol	1-propyl butyrate	sharp, pungent, rancid, sweaty, sickening
n-butyryl CoA (C4:0)	1-butanol	1-butyl butyrate	fruity, pineapple
2-butyryl CoA (C4:0)	1-butanol	1-butyl-2-butyrate <sup>a</sup>	fruity, apple, banana and pineapple
n-crotonoyl CoA (C4:1)	1-butanol	1-butyl crotoate <sup>a</sup>	n.d.
n-hexanoyl CoA (C6:0)	1-propanol	1-propyl hexanoate <sup>a</sup>	wine-like, cheese
n-decanoyl CoA (C10:0)	1-butanol	1-butyl decanoate <sup>a</sup>	Brandy (Whisky-Cognac)-like odor
benzoyl CoA (C7:0)	1-butanol	1-butyl benzoate <sup>a</sup>	mild floral-balsamic odor

**[0168]** Radio-GC analysis after incubations of the SLE27 (SAAT), SEQ ID NO: 1B recombinant enzyme with (<sup>14</sup>C)-acetyl-CoA and either octanol or hexanol showed the production of <sup>14</sup>C-labeled octylacetate and <sup>14</sup>C-labeled hexylacetate, respectively (Figure 11). The kinetic properties of SAAT were determined using (<sup>14</sup>C)-acetyl-CoA and five different alcohols. The apparent K<sub>m</sub> and V<sub>max</sub> for acetyl-CoA were 104.2 μM and 44.5 nmol product.h<sup>-1</sup>.μg<sup>-1</sup> protein, respectively. The apparent values of K<sub>m</sub> (in mM) and V<sub>max</sub> (in nmol product.h<sup>-1</sup>.μg<sup>-1</sup> protein) for octanol were 5.7 and 26.0, for hexanol 8.9 and 6.1, for butanol 46.1 and 4.2, for *trans*-2-hexenol 16.8 and 10.5 and for *cis*-2-hexenol 17.9 and 6.0, respectively. Activity was also detected with aromatic (benzyl- and phenylethyl-) and cyclic (furfuryl-) alcohols though activities were low compared to 1-octanol (4 to 10%). In contrast, no activity could be detected with the terpene alcohol linalool.

## Example 5

### Verification of alcohol acyl transferase activity of the proteins from melon, strawberry vesca, banana, apple, mango and lemon

**[0169]** The alcohol acyl transferase activity of the proteins having the sequences SEQ ID NO: 19 to 26, derived from strawberry vesca, banana, apple, mango, lemon (3x) and melon, respectively, was established in a similar way as described for strawberry protein encoded by SEQ ID NO: 1 in Example 4. From an overnight culture of *E. coli*, 0.75 ml was transferred into a 2 ml glass crimped vial, 0.3 g CaCl<sub>2</sub> was added and a small magnetic stirrer was put into the vial. The vial

was closed with a crimp cap and incubated during 15 min. at 50°C. The headspace of the vial was sampled with a 100 micro PDMS solid phase microextraction (SPME) device, supplied by Supelco, during this period. The SPME fiber was transferred into the injection port of a Fisons G8600 gas chromatograph and desorbed at 250°C during 10 seconds. The bottom split was closed during this period of 10 seconds and reopened afterwards. An HP-5 column with a 50m length, 0.32 mm ID and 1.05 micron film thickness was used. The oven was programed for 2 min. at 80°C, ramp of 8°C /min. to 250°C, for 5 min. at a head pressure of 47 kPa. An MD 800 mass spectrometer was used for the detection and identification of volatile esters, and the resulting mass spectra from M/z 35 to 400 were compared with the NIST database for identification. Where possible, retention times were compared to that of authentic reference compounds, obtained from Sigma or Aldrich.

**[0170]** The chromatograms obtained are shown in Figs. 12-18, of which Fig. 12 shows the results of using SEQ ID NO: 1 for comparison. The data obtained by the chromatograms are summarized in the following tables 3 to 9, respectively.

**Table 3.**

[0171] Shows ester formation (A) *E. coli* cell cultures expressing the strawberry alcohol acyl transferase (SLE27, SEQ ID NO: 1). (B) *E. coli* cell cultures expressing the empty vector cassette. Cultures supplemented with 1-butanol (Fig. 12).

Peak number	Retention time	Ester formed
1	6.797	butanoic acid, isopropyl ester acetic acid, butyl ester
2	7.027	propionic acid, butyl ester
3	9.027	ester, not identified
4	10.048	butanoic acid, butyl ester
5	11.028	hexanoic acid, butyl ester
8	15.368	acetic acid, benzyl ester
7	15.068	pentanoic acid, butyl ester
6	13.248	butanoic acid, phenylmethyl ester
9	17.278	

**Table 4.**

**[0172]** Shows ester formation (A) *E. coli* cell cultures expressing the melon alcohol acyl transferase (MAY 5, SEQ ID NO: 10). (B) *E. coli* cell cultures expressing the empty vector cassette. No alcohol supplements (Fig. 13).

Peak number	Retention time	Ester Formed
1	6.267	acetic acid, isobutyl ester
2	7.047	acetic acid, butyl ester
3	8.557	unknown ester
4	9.037	propionic acid, butyl ester
5	9.167	acetic acid, pentyl ester
6	10.048	unknown ester
7	10.438	acetic acid, 2-ethyl butyl ester
8	11.038	butanoic acid, butyl ester
9	11.408	acetic acid, hexyl ester
10	15.078	acetic acid, benzyl ester
11	15.378	hexanoic acid, butyl ester
12	17.048	acetic acid, phenyl ethyl ester
13	17.088	formic acid, unknown ester
14	17.298	butanoic acid, phenyl ethyl ester

**Table 5.**

**[0173]** Shows ester formation **(A)** *E. coli* cell cultures expressing the apple alcohol acyl transferase (MAY 3, SEQ ID NO: 11). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with isoamylalcohol, n-butanol, cis-2-hexen-1-ol and isopropanol(Fig. 14).

Peak number	Retention time	Ester formed
1	11.038	butanoic acid, butyl ester
2	17.089	formic acid, unknown ester

**Table 6.**

**[0174]** Shows ester formation **(A)** *E. coli* cell cultures expressing the citrus alcohol acyl transferase (CLF26: SEQ ID NO: 2). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with 1-butanol (Fig. 15).

Peak number	Retention time	Ester formed
1	7.047	acetic acid, butyl ester
2	10.086	propanoic acid, 2 methyl, butyl ester

**Table 7.**

**[0175]** Shows ester formation **(A)** *E. coli* cell cultures expressing the banana alcohol acyl transferase (MAY 2, SEQ ID NO: 12). **(B)** *E. coli* cell cultures expressing the empty vector cassette. No alcohol supplements (Fig. 16).

Peak number	Retention time	Ester formed
1	17.089	formic acid, unknown ester

**Table 8.**

**[0176]** Shows ester formation **(A)** *E. coli* cell cultures expressing the apple alcohol acyl transferase (MAY 3, SEQ ID NO: 11). **(B)** *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with isoamylalcohol, n-butanol, cis-2-hexen-1-ol and isopropanol (Fig. 17).

Peak number	Retention time	Ester formed
1	11.268	acetic acid, 1,2-hexenyl ester
2	15.268	butanoic acid, 1, 2-hexenyl ester

**Table 9.**

**[0177]** Shows ester formation (**A**) *E. coli* cell cultures expressing the strawberry vesca alcohol acyl transferase (SUN1, SEQ ID NO: 13 (**B**) *E. coli* cell cultures expressing the empty vector cassette. Both cultures supplemented with isoamylalcohol, n-butanol, cis-2-hexen-1-ol and isopropanol (Fig. 18).

Peak number	Retention time	Ester formed
1	6.977	acetic acid, butyl ester
2	8.287	acetic acid, iso-amyl ester
3	11.278	acetic acid, 2 hexenyl ester

Sequence listing

INFORMATION FOR SEQ ID NO: 1 (SLE27)